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REGULATION AND PHYSIOLOGICAL SIGNIFICANCE OF DAILY CYCLES  
IN GONADOTROPIN HORMONE LEVELS IN THE FEMALE GOLDFISH

by



ALICE HONTELA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH  
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## ABSTRACT

Changes in the patterns of the daily cycles in serum gonadotropin hormone (GTH) levels, and in some experiments, pituitary GTH levels in female goldfish subjected to long photoperiod and warm temperature, or short photoperiod and cold temperature for various lengths of time in winter and spring were determined. The effects of an outdoor pond regime, phase shifting the photoperiod and feeding times, diurnal temperature regimes, pinealectomy, blinding, and melatonin treatment on the daily cycles in serum GTH levels were also investigated. Ovarian condition was assessed to allow correlation with GTH levels.

In the stability experiments, fish were subjected to 12 hours of light and 12 hours of dark (12L12D) and 12<sup>0</sup> C for 6 to 8 and 30 to 32 days, or 16L8D/20<sup>0</sup> C for 5 to 7, 11 to 13, and 30 to 32 days in November and March. In these and all the other laboratory experiments, except in the phase shifting experiments, lights came on at 0600h, and fish were fed daily at 1000h and 1600h. Pituitaries and/or blood samples were collected throughout the 24 hour period, and GTH levels were determined by radioimmunoassay. The gonosomatic index (GSI) was determined for each fish and ovaries were examined histologically. Relatively low, uniform serum GTH levels were found throughout the day in fish kept under 12L12D/12<sup>0</sup> C for up to 32 days in November, but significant fluctuations persisted for at least 32 days in fish kept under this regime in March. In fish subjected to 16L8D/20<sup>0</sup> C, a peak in serum GTH levels was detected early in the photophase after 5 to 7 days in November and 11 to 13 days in March. Serum GTH levels were relatively high and uniform throughout the day after 30 to 32 days in November and March.

Significant daily fluctuations in pituitary GTH levels were found only in the group subjected to 16L8D/20<sup>0</sup> C for 5 to 7 days in November.

Fish were subjected to the outdoor pond regime for 12 days in September, April and May; they were fed daily at 1000h and 1600h. The patterns of the daily cycles in serum GTH levels were similar to the cycles found in fish kept under similar but constant conditions of photoperiod and temperature in the laboratory.

In the photoperiod and feeding phase shifting experiments, fish were kept under 16L8D/20<sup>0</sup> C in November and April, and the onset of light and/or feeding times were shifted by several hours in the experimental groups. Photoperiod and feeding entrained significant fluctuations in serum GTH levels when the onset of light and the first daily feeding were 4 hours apart, but not when they were 10 hours apart. Fish were subjected to 16L8D for 14 to 16 days in February, and either a constant warm (20<sup>0</sup> C) or a diurnal sinusoidal (12 to 20<sup>0</sup> C) temperature regime, the warmth being imposed during photophase or scotophase. While relatively high, uniform serum GTH levels were found throughout the 24 hour period in fish subjected to constant warmth, warm temperature during the day promoted fluctuations in serum GTH levels, and warmth during night resulted in relatively low, uniform serum GTH levels.

In another series of experiments, control, pinealectomized and/or blinded fish were subjected to the 16L8D/20<sup>0</sup> C or 8L16D/20<sup>0</sup> C regime. Pinealectomy had no effect on serum GTH levels in sexually regressed fish subjected to 16L8D/20<sup>0</sup> C in early fall, but promoted fluctuations in fish under 8L16D/20<sup>0</sup> C in March. Pinealectomy and/or blinding suppressed fluctuations in serum GTH levels in fish exposed to 16L8D/20<sup>0</sup> C



in March and April.

Melatonin (1  $\mu$ g, 10  $\mu$ g or 12  $\mu$ g/g body weight) was administered to pinealectomized or control fish held under 16L8D/20<sup>0</sup> C in November or the spring months; injections were given in the morning or the afternoon. Morning melatonin injections lowered the early-photophase peak in serum GTH levels in intact fish in November, but not in February. Afternoon injections had no effect on serum GTH levels in pinealectomized or control fish in the spring, or control fish in November.

In most experiments, abolition of the daily fluctuations in serum GTH levels was correlated with lower GSI's or a greater proportion of severely atretic oocytes in the ovaries, compared to groups in which the serum GTH levels fluctuated throughout the 24 hour period.

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## GENERAL INTRODUCTION

Temperate zone teleost fishes exhibit well defined cycles of reproductive activity. Gonadal development in these fishes is controlled by the hypothalamo-hypophysial axis through mechanisms similar to those in higher vertebrates. The existence of a gonadotropin releasing hormone (GRH) which is produced by the hypothalamus and regulates the release of gonadotropin hormones (GTH) by the pituitary is well established (for review, see Peter, 1982). Furthermore, it has been proposed recently that a gonadotropin release-inhibitory factor (GRIF) is present in the goldfish (Peter *et al.*, 1978a; Peter and Paulencu, 1980). Good evidence for the trophic role of pituitary GTH in gonadal growth has accumulated over the years (for review, see Peter and Crim, 1979; Peter, 1981; Billard *et al.*, 1982). Although earlier studies suggested that only one GTH, similar to both mammalian luteinizing hormone (LH) and follicle stimulating hormone (FSH), exists in teleosts (for review, see Peter and Crim, 1979), two GTH's have been recently detected in four species of teleosts, including the common carp (Ng and Idler, 1979; Idler and Ng, 1979). The two hormones differ in their carbohydrate content, and some of their actions (for review, see Peter, 1981). Although both the GTH rich in carbohydrate content (Con A-II/GTH) and the GTH low in carbohydrate content (Con A-I/GTH) are required for vitellogenesis, oocyte maturation and ovulation are stimulated only by the Con A-II/GTH; this GTH also promotes spermiation, c-AMP activity in the gonads, and steroidogenesis. Only the carbohydrate rich GTH can be measured by RIA at present, and therefore all the literature and data discussed in this manuscript pertain to



this GTH. Sex steroid hormones synthesized by the gonadal tissue (for review, see Nagahama *et al.*, 1982) have been identified in several teleost species (Azoury and Eckstein, 1980; Campbell *et al.*, 1980; Kime, 1980; Ng and Idler, 1980; Fostier *et al.*, 1978), and are also required for the maturation and maintenance of the gonads, and ovulation or spermiation (e.g. Duffey and Goetz, 1980; Sundararaj and Nath, 1981; Billard *et al.*, 1982). The sex steroids also modulate hypothalamic and pituitary secretory activity (e.g. Billard, 1978; Kim *et al.*, 1978; Olivereau and Olivereau, 1979; Ueda and Takahashi, 1980; Crim *et al.*, 1981), providing a feedback link between the gonads and hypothalamo-hypophysial axis.

While these mechanisms constitute the basic internal regulatory system, environment cues such as photoperiod and temperature seem to constitute a major external input into the reproductive axis of some teleost fishes (for review, see de Vlaming, 1972, 1974; Peter, 1981). Since the development of highly specific radioimmunoassays for the GTH (Con A-II/GTH) of some species of Cyprinidae and Salmonidae (Breton *et al.*, 1972, 1976; Crim *et al.*, 1973, 1975, 1976), GTH levels have been determined in fishes subjected to a variety of experimental regimes. It has been shown that blood or pituitary GTH levels fluctuate throughout the season, and are influenced by environmental cues (for review, see Billard *et al.*, 1978; Billard and Breton, 1978; Peter and Hontela, 1978; Peter and Crim, 1979; Peter, 1981). From these early studies, it was proposed (Crim *et al.*, 1975; Breton *et al.*, 1975; Breton and Billard, 1977) that as the gonads mature, blood levels of GTH gradually increase, from a low level detected in immature fish to a higher level

found in mature fish. It was hypothesized that progressive development of the gonads requires progressive increases in the blood levels of GTH.

In addition to seasonal cycles in blood levels of GTH, GTH levels in the pituitary (O'Connor, 1972) and the blood (Breton *et al.*, 1972; Vodcnik *et al.*, 1978) have also been found to fluctuate throughout the day. The relatively large changes detected within a 24 hour period in these studies suggested that such variations might be physiologically important. Daily cycles in the serum GTH levels in the goldfish, and the effects of photoperiod, temperature and sexual condition on these cycles were therefore investigated in a recent study (Hontela and Peter, 1978 ). GTH levels throughout a 24 hour period were low and relatively uniform in fish with regressed gonads under all experimental photoperiod and temperature regimes, whereas significant fluctuations were usually detected in females undergoing ovarian recrudescence or in females that had completed ovarian recrudescence. The patterns of the daily variations in serum GTH levels in the latter two groups were similar under the same photoperiod and temperature regime. Furthermore, fish at various stages of gonadal development held under the same photoperiod and temperature regime had similar low serum GTH levels at certain times of the day. The data suggested that as the gonads mature in the goldfish, the pattern of the daily cycle in serum GTH levels changes from one characterized by low levels and small or no fluctuations to one with fluctuations of a higher magnitude.

In the present study, the hypothesis that the daily cycles in serum GTH levels are of importance for gonadal development in the goldfish was tested, and the daily cycles were further characterized. The cycles were manipulated through various treatments and the effect of these

manipulations on the ovarian development was investigated.

The temporal changes in the pattern of the GTH cycle under long photoperiod and warm temperature, and under short photoperiod and cold temperature, and the physiological significance of these changes are discussed in the first chapter of the present study. The effects of a long term exposure to a particular photoperiod and temperature regime on the pattern of the GTH cycle and on gonadal development were investigated since the long term and the day to day stability of the cycles was not known. Since the effects of a gradually changing natural photoperiod and temperature on the cycle are unknown, attempts were also made to assess the daily cycle in serum GTH levels in fish held in an outdoor pond.

The effects of temperature, photoperiod and time of feeding on GTH cycles and gonadal development are discussed in the second chapter. Both photoperiod and temperature influence the daily cycles in serum GTH levels in the goldfish (Hontela and Peter, 1978 ); however, the relative importance of each of these factors has not been determined. Furthermore, the influence of the time of feeding on the GTH cycle has not been investigated. Experiments involving phase shifting the photoperiod and feeding times were done in order to investigate the synchronizing effect of these cues on the daily GTH cycle. Also, since goldfish might experience a daily temperature cycle in small ponds, the influences of such cycles on daily GTH fluctuations and ovarian development were determined.

One of the mechanisms by which light might affect GTH cycles and gonadal development is investigated in the third chapter. The pineal



organ of teleost fishes is photosensory and has some characteristics of an endocrine gland (for review, see Reiter, 1979, 1980a). It influences gonadal development in teleost fishes (Fenwick, 1970a; Urasaki, 1972, 1976; de Vlaming, 1975; Vodcnik *et al.*, 1978); however, there has been only one investigation of the role of the pineal in regulation of GTH levels (Vodcnik *et al.*, 1978). The effects of pineal-ectomy, blinding and sexual condition on daily cycles in serum GTH are discussed in Chapter 3.

Little is known about the mechanisms by which the pineal influences GTH levels in teleost fishes. At present, melatonin is considered to be a major secretory product of the pineal organ of many vertebrates (Reiter, 1979, 1980a). It has been shown to retard gonadal development in several teleosts (Fenwick, 1970b; Urasaki, 1972; Sundararaj and Keshavanath, 1976; Saxena and Anand, 1977; Borg and Eckström, 1981) but the effects of melatonin on GTH levels have not been investigated. The effects of melatonin administration on GTH levels are discussed in the fourth chapter.



## CHAPTER 1: TEMPORAL CHANGES IN THE PATTERNS OF DAILY GONADOTROPIN HORMONE CYCLES

### INTRODUCTION

Blood gonadotropin hormone (GTH) levels fluctuate significantly throughout the day in goldfish, *Carassius auratus* (Breton *et al.*, 1972; Vodick *et al.*, 1978; Hontela and Peter, 1978, 1980) undergoing ovarian recrudescence or that have completed ovarian recrudescence, when exposed to specific sets of environmental conditions. Hontela and Peter hypothesized that the daily cycles in serum GTH levels are important for gonadal development in the goldfish since daily fluctuations were usually found in maturing and mature females, but not in sexually regressed animals. Also, both the patterns of the daily GTH cycles and gonadal development were influenced by photoperiod and temperature.

Fluctuations in GTH levels throughout the day occur also in other vertebrates. The large preovulatory LH surge has been detected in representatives of all vertebrate classes. Pulses in LH and/or FSH levels of smaller magnitude than the preovulatory LH surge, either continuous throughout the day or at only certain times of the day have been reported in birds (Hashigushi *et al.*, 1977a, 1977b; Scanes *et al.*, 1978, 1980; Balthazart *et al.*, 1981; Wingfield *et al.*, 1981), several species of rodents (Seegal and Goldman, 1975; Turek *et al.*, 1976; Hostetter and Piacsek, 1977; Gallo, 1980, 1981; Kimura *et al.*, 1981), pigs (Brinkley, 1981), sheep (Lincoln and Peet, 1977; Walton *et al.*, 1980; Kennaway *et al.*, 1981) and monkeys (Puri *et al.*, 1980). Several other variables of the hypothalamo-hypophysial-gonadal axis, in addition to GTH levels also

fluctuate diurnally. Daily fluctuations in the luteinizing hormone-releasing hormone (LHRH) content of various parts of the brain (Kalra and Kalra, 1978; Tuominen *et al.*, 1979; Szafarczyk *et al.*, 1980; Wenger and Leonardelli, 1980; Kerdelhue *et al.*, 1981), pituitary sensitivity to LHRH (Wilkinson and Moger, 1981) and plasma levels of sex steroids (Allen and Bradshaw, 1980; Puri *et al.*, 1980; Miyatake *et al.*, 1980; Brinkley, 1981) have been described in several mammals. The gonadal responsiveness to GTH varies throughout the day in man (Nankin *et al.*, 1980), mouse (Lamond and Braden, 1959), rat (Grizzard *et al.*, 1978), chicken (Dusseau and Boscher, 1976), frog (Easley *et al.*, 1979), and also in some teleost fishes (de Vlaming and Vodicnik, 1977; Kuo and Watanabe, 1978; Peter *et al.*, 1982). Apparently, fluctuating hormone concentrations are essential for the maintenance of physiologically responsive receptors in some target tissues (for review, see Catt *et al.*, 1979); evidence that exposure to constant high levels of GTH desensitizes the mammalian gonad is accumulating (e.g. Jääskeläinen *et al.*, 1980; Chan *et al.*, 1981; Sen *et al.*, 1979). However, such investigations have not been carried out in submammalian vertebrates.

The reproductive axis of teleosts represents a unique system for study of the significance of fluctuating blood levels of GTH on gonadal development. In the goldfish it is possible to correlate periods of greater fluctuations in serum GTH and of heightened gonadal activity (Hontela and Peter, 1978, 1980). The only other vertebrates in which such a correlation can be made at present are the Japanese quail (Hashiguchi *et al.*, 1977a) and the sheep (Lincoln, 1976; Lincoln and Peet, 1977). In the present study, the daily fluctuations in serum GTH levels in goldfish exposed to several photoperiod and temperature regimes,

including an outdoor pond regime, for various lengths of time, were determined. In addition, the ovarian condition of the fish was examined by histology to allow correlation with GTH levels. This study also provided data on the long and short term stability of the daily cycles in serum GTH levels under various photoperiod and temperature regimes, and made possible a comparison of the patterns of the GTH cycles in fish held under laboratory conditions and a natural outdoor regime.

## MATERIALS AND METHODS

### I. Experimental animals

Goldfish, *Carassius auratus* (common or comet variety, standard body length 6.5 - 8.0 cm) were purchased from Grassyfork Fisheries Co., Inc., Martinsville, Indiana at various times of the year. Fish were sexed upon arrival by external examination and females were selected for the experiments.

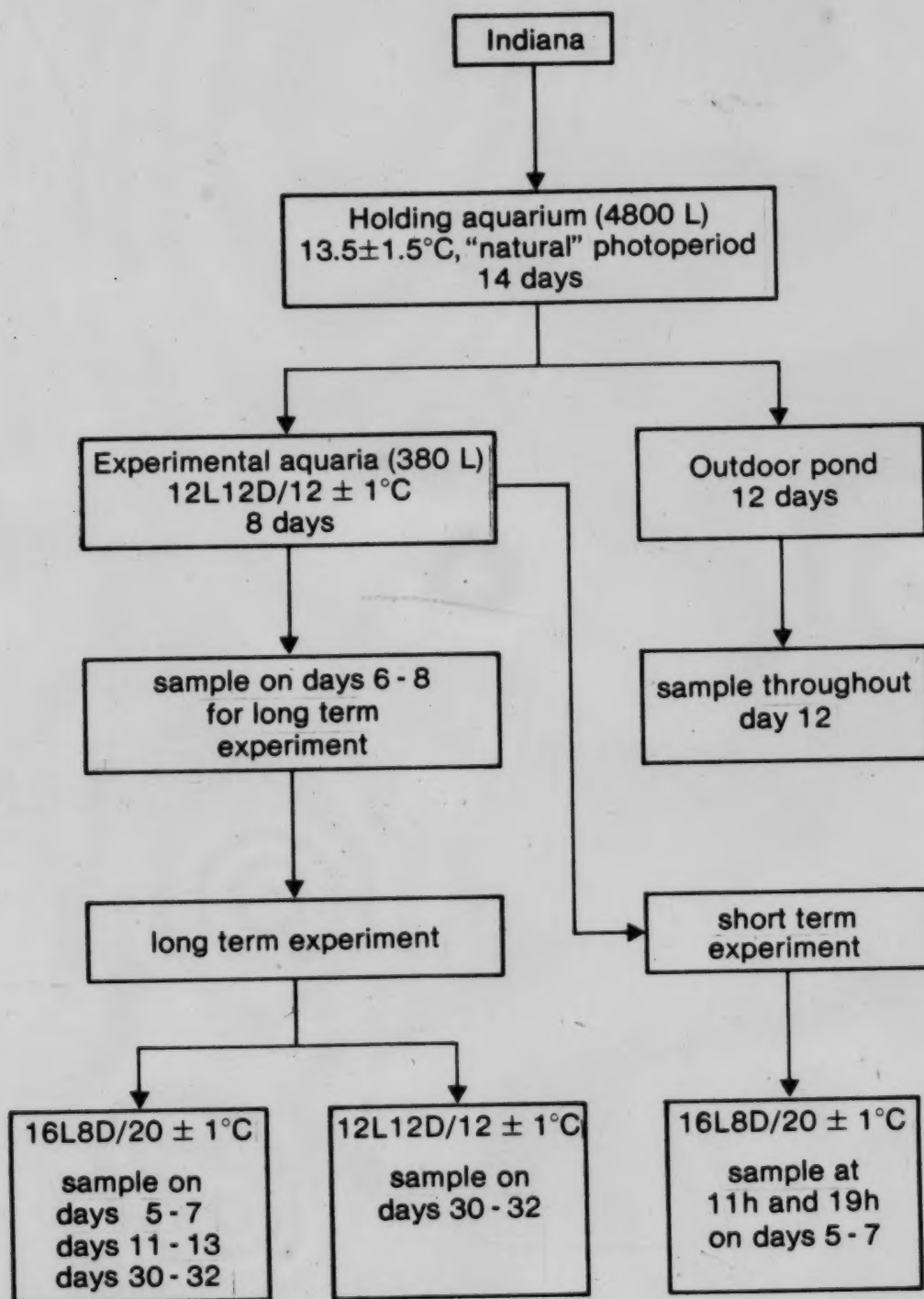
### II. Environmental maintenance regime

Fish were held in a flow-through aquarium (4800 L) maintained at  $13.5 \pm 1.5^{\circ}\text{C}$ ; room lights were regulated to simulate the length of the natural Edmonton photoperiod. The animals were fed an excess of commercial fish food (Ewos, Astra Chemicals Ltd., Mississauga, Ontario) at 1000h and 1600h. This environmental regime, referred to in the text as the initial acclimation period, was imposed for 14 days. A diagrammatic outline of the protocol is presented in Figure 1.1.

Figure 1.1. Diagrammatic outline of the maintenance and sampling regimes used in the stability experiments. |







### Long and short term experiments

Following the initial acclimation period, fish were transferred into experimental flow-through aquaria (380 L, 56 fish/aquarium) maintained at  $12 \pm 1^{\circ}\text{C}$  and a photoperiod of 12 hours light and 12 hours darkness (12L12D) for 8 days. The light phase started at 0600h (one 15 watt, cool white fluorescent light was used per aquarium). Then, the experimental regime of 16L8D/20  $\pm 1^{\circ}\text{C}$  (lights on at 0600h) was imposed (designated as day 1, see Figure 1.1), or the fish were left under 12L12D/12  $\pm 1^{\circ}\text{C}$ .

### Outdoor experiments

Following the initial acclimation period, 70 fish were transferred to an outdoor pond (maximum depth = 0.7 m, volume of water about 4500 L) and subjected to the natural photoperiod and temperature regime for 12 days.

### III. Blood samples

Blood was withdrawn from the caudal vasculature, and serum collected and stored as described by Hontela and Peter (1978). Each fish was sampled once and killed after the procedure. Gonadosomatic index (GSI) was calculated as follows: 
$$\frac{\text{weight of ovaries}}{\text{total body weight}} \times 100\%$$
; a section of the ovaries was fixed in Bouin's solution for histology. In some experiments, the pituitary gland was dissected out, frozen on dry ice and stored at  $-27^{\circ}\text{C}$ , prior to lyophilization, weighing and homogenization in barbital buffer (pH = 8.6).

### Sampling schedule

During each sampling period, fish were sampled throughout three consecutive days at 0700h and 1900h on the first day, at 0300h and 1500h on the second day, and 1100h and 2300h on the third day. This sampling schedule will be referred to hereafter as the standard sampling times.

In the long term stability experiments, the sampling periods were days 6, 7, 8 and 30, 31, 32 of the 12L12D/12  $\pm$  1 $^{\circ}$  C regime, and days 5, 6, 7, and 11, 12, 13 and 30, 31, 32 of the 16L8D/20  $\pm$  1 $^{\circ}$  C regime. Experiments in which this protocol was used were carried out in November with fish in early ovarian recrudescence ("recrudescent" females), and in March with fish having ovaries containing some oocytes that had completed vitellogenesis ("mature" females).

In the short term stability experiments, fish were sampled at 1100h and 1900h on days 5, 6, and 7 of the 16L8D/20  $\pm$  1 $^{\circ}$  C regime in December.

In the outdoor experiments, fish were sampled at 0700h, 1100h, 1500h, 1900h, 2300h and 0300h on day 12 of the natural photoperiod and temperature regime. At the completion of the sampling, 2 to 10 excess fish remaining in the pond were sacrificed. This protocol was followed in September, April and May.

### IV. Radioimmunoassay for GTH

The serum samples and pituitary homogenates were assayed with a radioimmunoassay specific for carp GTH. The assay and its characteristics are described in Crim *et al.* (1976) and Hontela and Peter (1978, 1980).

## V. Histology

Histological examination of the ovaries was carried out as described by Hontela and Peter (1978) with the following modifications: oocytes in the first growth stage were not counted; oocytes (diameter = 350 to 900  $\mu$ ) which contained yolk globules in the inner part of the cytoplasm and yolk vesicles in the outer two-thirds of the cytoplasm were classified as being in the primary and secondary yolk stage. Oocytes (diameter = 1000  $\mu$ ) containing yolk vesicles only in few peripheral rows of the cytoplasm while the inner part of the cytoplasm was filled with yolk globules were classified as being in the tertiary yolk stage. Ovaries in which 3 or more atretic follicles could be observed in the field of vision (2.5 mm<sup>2</sup>) were classified as severely atretic.

## VI. Statistical analysis

Analysis of variance and Duncan's multiple range test at  $p < 0.05$  (Steel and Torrie, 1960) or Student's "t"-test at  $p < 0.05$ , 0.01 were used for statistical comparisons of the GTH levels or of the GSI's. Student's "t"-test and the proportions comparison test (Walpole, 1968) were used in the analysis of the histological data.

## RESULTS

### I. Long term stability experiments

#### November

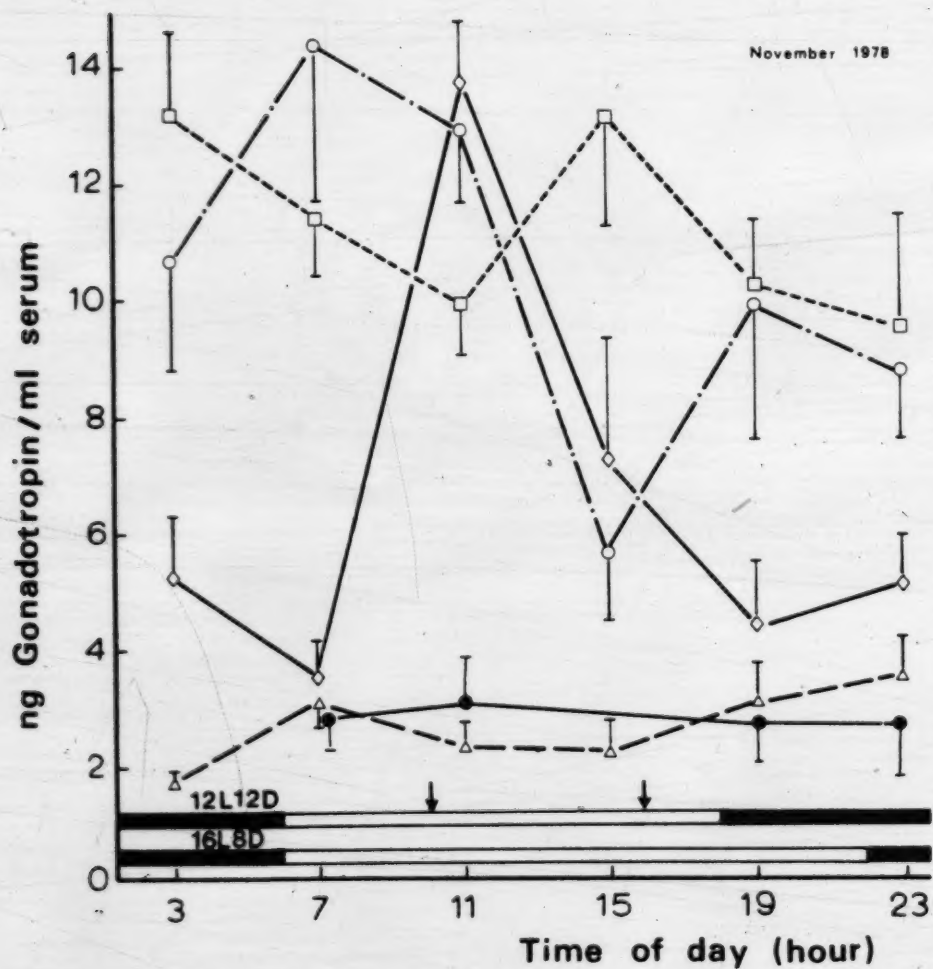
The serum levels found in fish in the long term acclimation experiment in November are presented in Figure 1.2. Low GTH levels throughout



Figure 1.2. The serum GTH levels (mean  $\pm$  SE) in fish in the long term acclimation experiment in November, 1978.

The results of the Duncan's multiple range test ( $p < 0.05$ ) are shown at the top of the figure. The feeding times (indicated by arrows) and the experimental photoperiod regimes are shown at the bottom; the black horizontal bars represent the dark phase of the photoperiod, the empty bars represent the light phase. The means are averages from 6 to 7 fishes.

|    |    |    |    |    |    |               |           |           |
|----|----|----|----|----|----|---------------|-----------|-----------|
| 3  | 15 | 11 | 19 | 7  | 23 | 12L 12D/ 12°C | Day 6- 8  | △ — — — △ |
| 7  | 19 | 23 | 3  | 15 | 11 | 16L 8D/ 20°C  | Day 5- 7  | ◇ — — — ◇ |
| 15 | 23 | 19 | 3  | 11 | 7  | 16L 8D/ 20°C  | Day 11-13 | ○ — — — ○ |
| 23 | 11 | 19 | 7  | 3  | 15 | 16L 8D/ 20°C  | Day 30-32 | □ — — — □ |
| 11 | 23 | 19 | 7  |    |    | 12L 12D/ 12°C | Day 30-32 | ● — — — ● |



the day and fluctuations of a magnitude less than 2 ng/ml of serum were found in fish held under 12L12D/12  $\pm$  1 $^{\circ}$  C for 6 to 8 days, or for an additional 30 to 32 days. In fish subjected to 16L8D/20  $\pm$  1 $^{\circ}$  C for 5 to 7 days, subsequent to 8 days under 12L12D/12  $\pm$  1 $^{\circ}$  C, a large peak ( $p < 0.05$ ) at 1100h and low GTH levels throughout the rest of the day were detected. After 11 to 13 days of exposure to 16L8D/20  $\pm$  1 $^{\circ}$  C, the highest levels were still found early in the day (0700h and 1100h); however, the GTH levels at other times of the day were somewhat increased compared to the group acclimated for 5 to 7 days, and no statistically significant fluctuations were found. Relatively high levels and no significant fluctuations were detected after 30 to 32 days of exposure to 16L8D/20  $\pm$  1 $^{\circ}$  C.

As shown in Figure 1.3, the pituitary GTH levels in fish held under 12L12D/12  $\pm$  1 $^{\circ}$  C for 6 to 8 days were greater ( $p < 0.05$ ) at 2300h than at other times of the day. Two peaks in pituitary GTH levels (at 0300h and 1900h) were detected in fish subjected to 16L8D/20  $\pm$  1 $^{\circ}$  C for 5 to 7 days; there was no significant correlation between pituitary and serum GTH levels at various times of the day for these fish, as shown in Figure 1.4. No significant fluctuations in pituitary GTH levels were found in fish subjected to 16L8D/20  $\pm$  1 $^{\circ}$  C for 11 to 13 days or 30 to 32 days (Figure 1.3).

Mean serum and pituitary GTH levels found in fish in the long term stability experiment in November are shown in Figure 1.5. For this analysis, the GTH levels from fish within an experimental group (e.g. the 16L8D/20  $\pm$  1 $^{\circ}$  C day 5 to 7 group) sampled at various times of the day were pooled and expressed as an average. The mean serum GTH level

Figure 1.3. The pituitary GTH levels (mean  $\pm$  SE) in fish in the long term acclimation experiment in November, 1978.

See caption of Figure 1.1 for more detail.



|    |    |    |    |    |    |
|----|----|----|----|----|----|
| 11 | 19 | 7  | 15 | 3  | 23 |
| 23 | 11 | 15 | 7  | 19 | 3  |
| 7  | 3  | 11 | 19 | 15 | 23 |
| 11 | 15 | 3  | 23 | 7  | 19 |

12L12D/12°C

16L8D/20°C

16L8D/20°C

16L8D/20°C

Day 6-8 Day 5-7 Day 11-13 Day 30-32 

November 1978

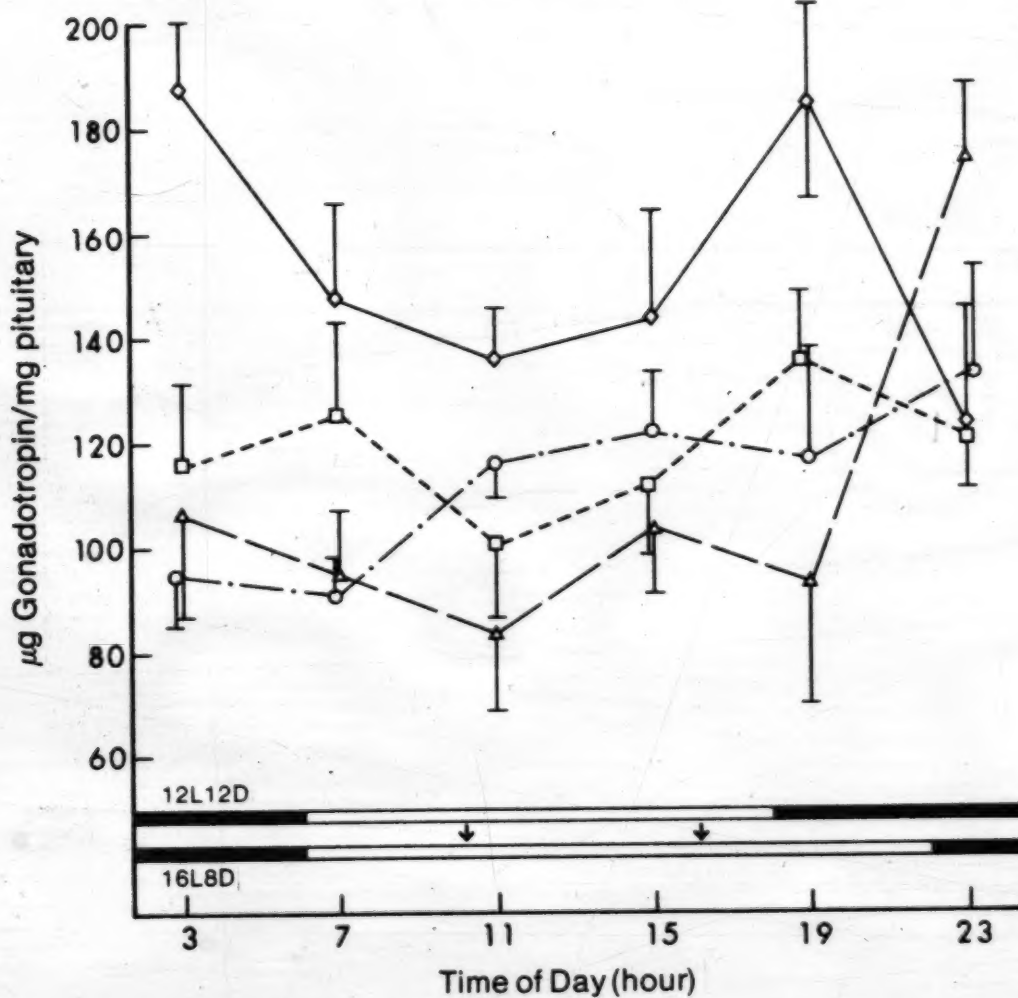


Figure 1.4. Serum and pituitary GTH levels (mean  $\pm$  SE) on days 5 to 7 in fish subjected to the 16L8D/20  $^{\circ}$ C regime in November.

Numbers of fish sampled at each time are indicated beside the means. The correlation between the serum and pituitary GTH levels was not significant ( $r = 0.1$ ;  $p < 0.05$ ). See caption of Figure 1.1 for more detail.

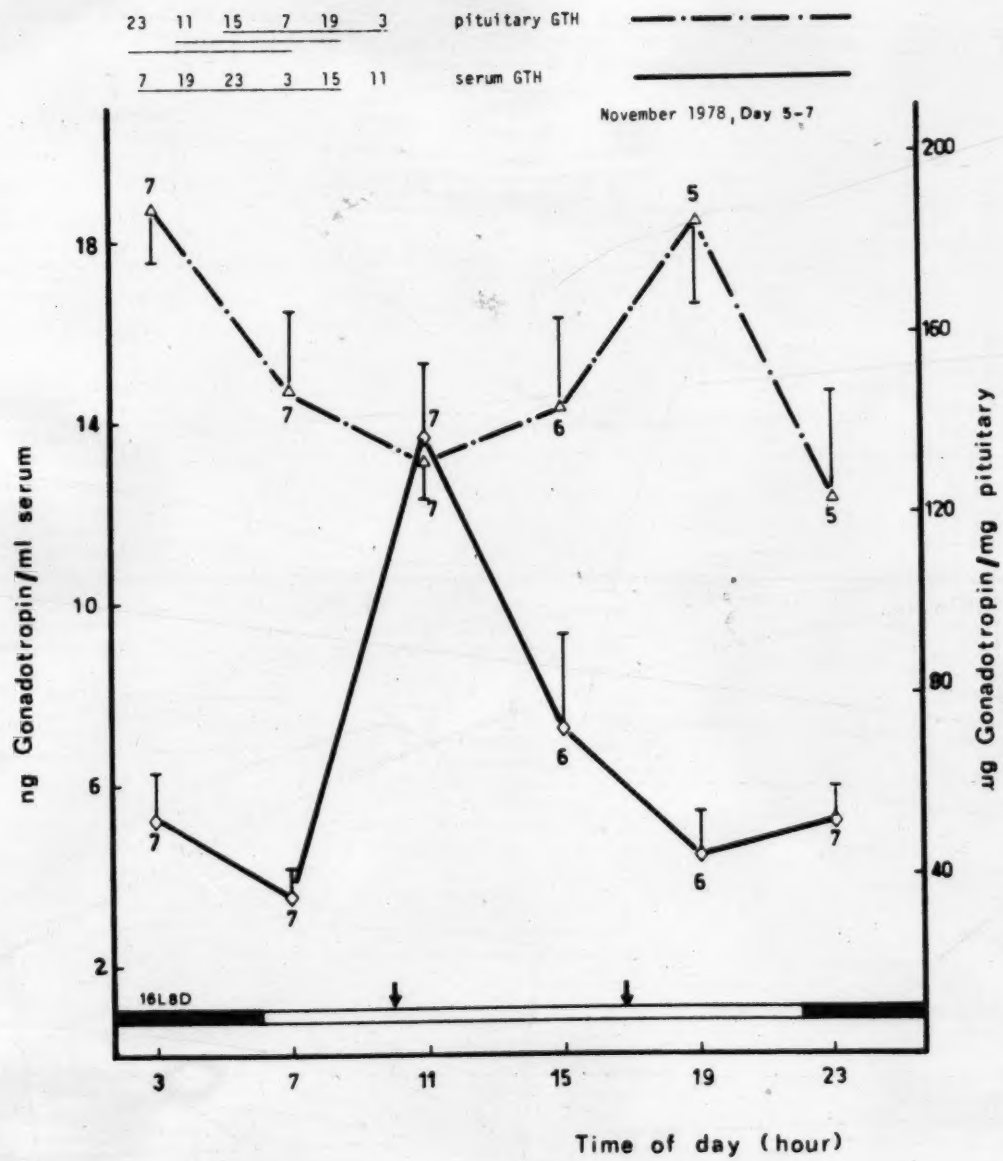
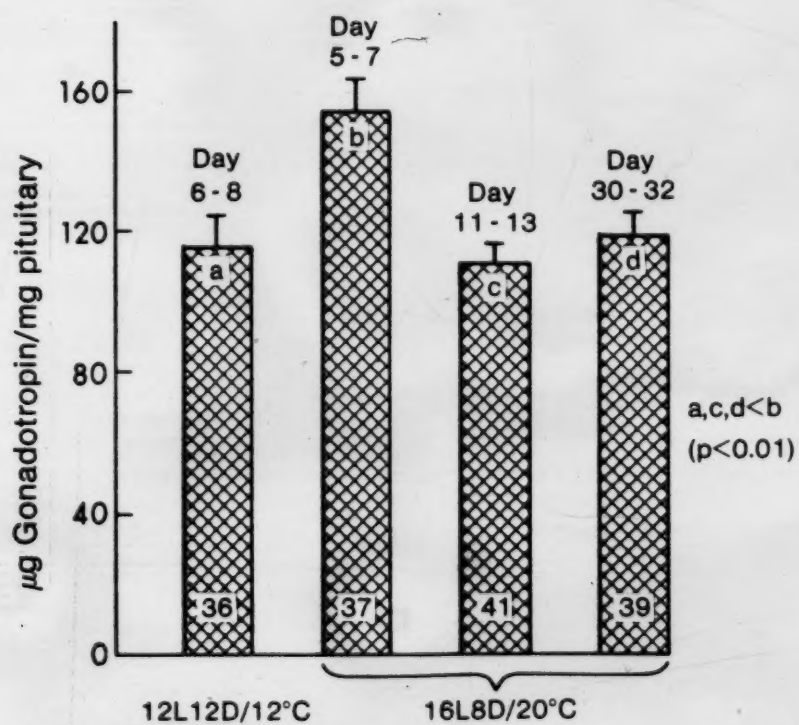
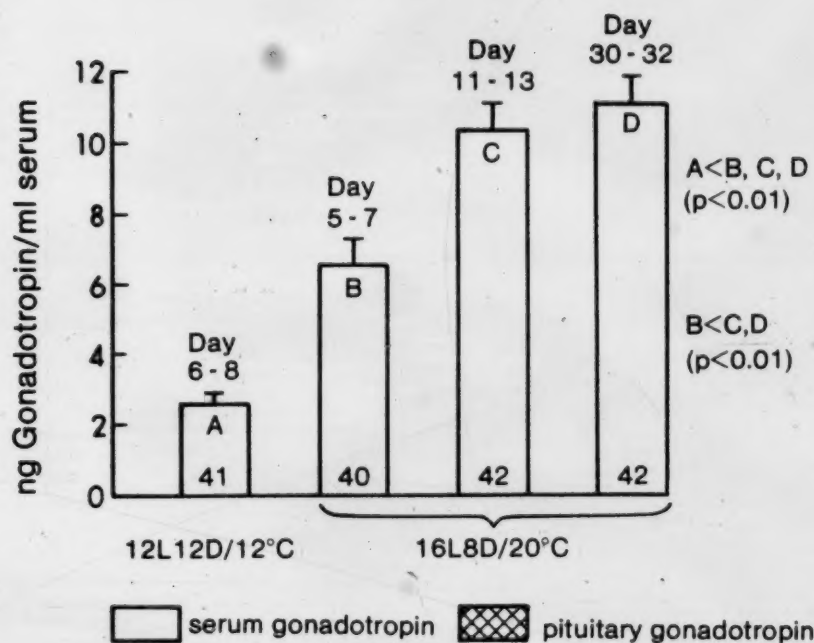


Figure 1.5. Mean serum and pituitary GTH levels (mean  $\pm$  SE) in fish in the long term acclimation experiment in November, 1978.

Numbers of fish sampled are shown at the bottom of the histogram bars. Significant differences by Student's t-test ( $p < 0.05$ ) are indicated at the right of the histograms.



November 1978



of the fish subjected to  $12L12D/12 \pm 1^{\circ}C$  for 6 to 8 days were lower ( $p < 0.01$ ) than the mean levels of the other three groups. Also, the mean serum GTH level of the group held under  $16L8D/20 \pm 1^{\circ}C$  for 5 to 7 days was lower ( $p < 0.01$ ) than the levels of the groups held under  $16L8D/20 \pm 1^{\circ}C$  for 11 to 13 days and 30 to 32 days. The mean pituitary GTH level of the group held under  $16L8D/20 \pm 1^{\circ}C$  for 5 to 7 days was higher ( $p < 0.01$ ) than the mean levels of the other three groups, which were not significantly different from each other.

The mean GSI values of the four experimental groups were not significantly different. However, histological examination of the cellular composition of ovaries from the four groups (Table 1.1) revealed that fish subjected to  $12L12D/12 \pm 1^{\circ}C$  for 6 to 8 days or 30 to 32 days, or to  $16L8D/20 \pm 1^{\circ}C$  for 30 to 32 days had fewer ( $p < 0.05$ ) oocytes in the  $1^{\circ}$  and  $2^{\circ}$  yolk stage than fish held under  $16L8D/20 \pm 1^{\circ}C$  for 11 to 13 days. Also, fish held under  $16L8D/20 \pm 1^{\circ}C$  for 30 to 32 days had fewer ( $p < 0.05$ ) oocytes in the  $1^{\circ}$  and  $2^{\circ}$  yolk stage than the group held under  $16L8D/20 \pm 1^{\circ}C$  for 5 to 7 days. Furthermore, 65% of ovaries examined in the group subjected to  $16L8D/20 \pm 1^{\circ}C$  for 30 to 32 days were classified as severely atretic whereas severely atretic ovaries were not observed in the other experimental groups ( $p < 0.001$ ). Figure 1.6.1 represents a normal ovary of a maturing fish subjected to  $16L8D/20 \pm 1^{\circ}C$  for 11 to 13 days in November; Figure 1.6.2 represents a severely atretic ovary of a fish kept under this regime for 30 to 32 days.

#### March

The serum GTH levels found in the fish in the long term stability experiments in March are shown in Figure 1.7 (March, 1979) and Figure 1.8 (March, 1981). In fish subjected to  $12L12D/12 \pm 1^{\circ}C$  for 6 to 8 days,

Table 1.1: Cellular composition of ovaries of fish in the long term stability experiments in November, 1978.

| Experimental Group                  | GSI<br>( $\bar{x} \pm SE$ )  | Number of cells/mm <sup>2</sup> of ovary ( $\bar{x} \pm SE$ ) |                    |                                              | Number of severely atretic ovaries<br>(% of total number in a group) |     |
|-------------------------------------|------------------------------|---------------------------------------------------------------|--------------------|----------------------------------------------|----------------------------------------------------------------------|-----|
|                                     |                              | Perinucleolus Stage                                           | Yolk Vesicle Stage | 1 <sup>0</sup> and 2 <sup>0</sup> Yolk Stage | 3 <sup>0</sup> Yolk Stage                                            |     |
| 12L12D/12 <sup>0</sup> C<br>Day 6-8 | 3.0±0.2<br>n=41              | 34.5±3.5<br>n=24                                              | 8.0±0.7            | 5.1±0.6 <sup>a</sup>                         | 0                                                                    | 0   |
|                                     | Day 30-32<br>3.2±0.3<br>n=21 | 36.6±4.2<br>n=15                                              | 9.4±1.3            | 5.0±0.7 <sup>b</sup>                         | 0                                                                    | 0   |
| 16L8D/20 <sup>0</sup> C<br>Day 5-7  | 3.1±0.1<br>n=40              | 35.5±3.1<br>n=22                                              | 7.3±0.5            | 6.4±0.5 <sup>c</sup>                         | 0                                                                    | 0   |
|                                     | Day 11-13<br>3.2±0.2<br>n=42 | 31.4±4.7<br>n=24                                              | 7.3±0.5            | 6.7±0.6 <sup>d</sup>                         | 0.02±0.01<br>(2 fish)                                                | 0   |
|                                     | Day 30-32<br>3.4±0.3<br>n=42 | 36.3±4.5<br>n=23                                              | 6.7±0.7            | 5.0±0.4 <sup>e</sup>                         | 0.09±0.08<br>(3 fish)                                                | 65* |

d > a,b,e (p < 0.05); c > e (p < 0.05); \*greatest proportion of severely atretic ovaries found in this group (p < 0.001)

Figure 1.6. Ovaries in fish in the early stages of ovarian recrudescence (November) subjected to the 16L8D/20<sup>0</sup> C regime (X100).

1) Normal ovary                      2) Severely atretic ovary

p = oocyte in the perinucleolus stage

v = oocyte in the yolk vesicle stage

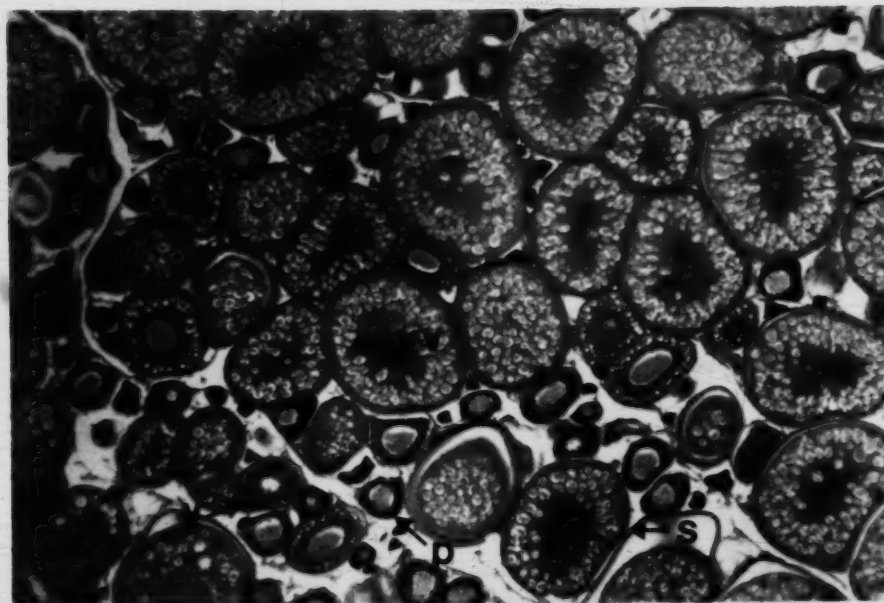
s = oocyte in the 1<sup>0</sup> and 2<sup>0</sup> yolk stage

a = atretic ovary

yv = yolk vesicles



1)



2)

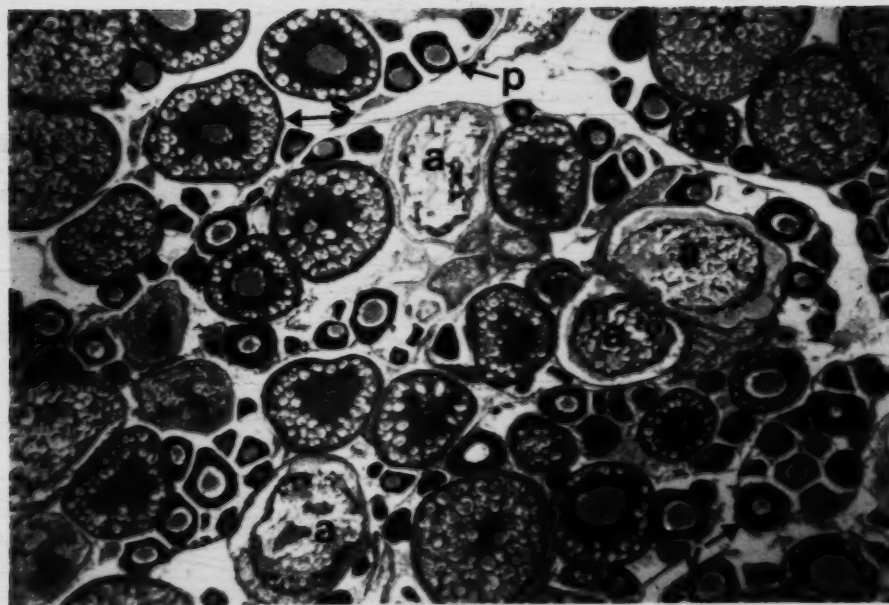


Figure 1.7. The serum GTH levels (mean  $\pm$  SE) in fish in the long term acclimation experiment in March, 1979.

See caption of Figure 1.1 for more details.

15 23 7 3 19 11

3 7 19 15 11 23

19 3 23 7 15 11

23 19 15 11 7

12L12D/12°C

16L8D/20°C

16L8D/20°C

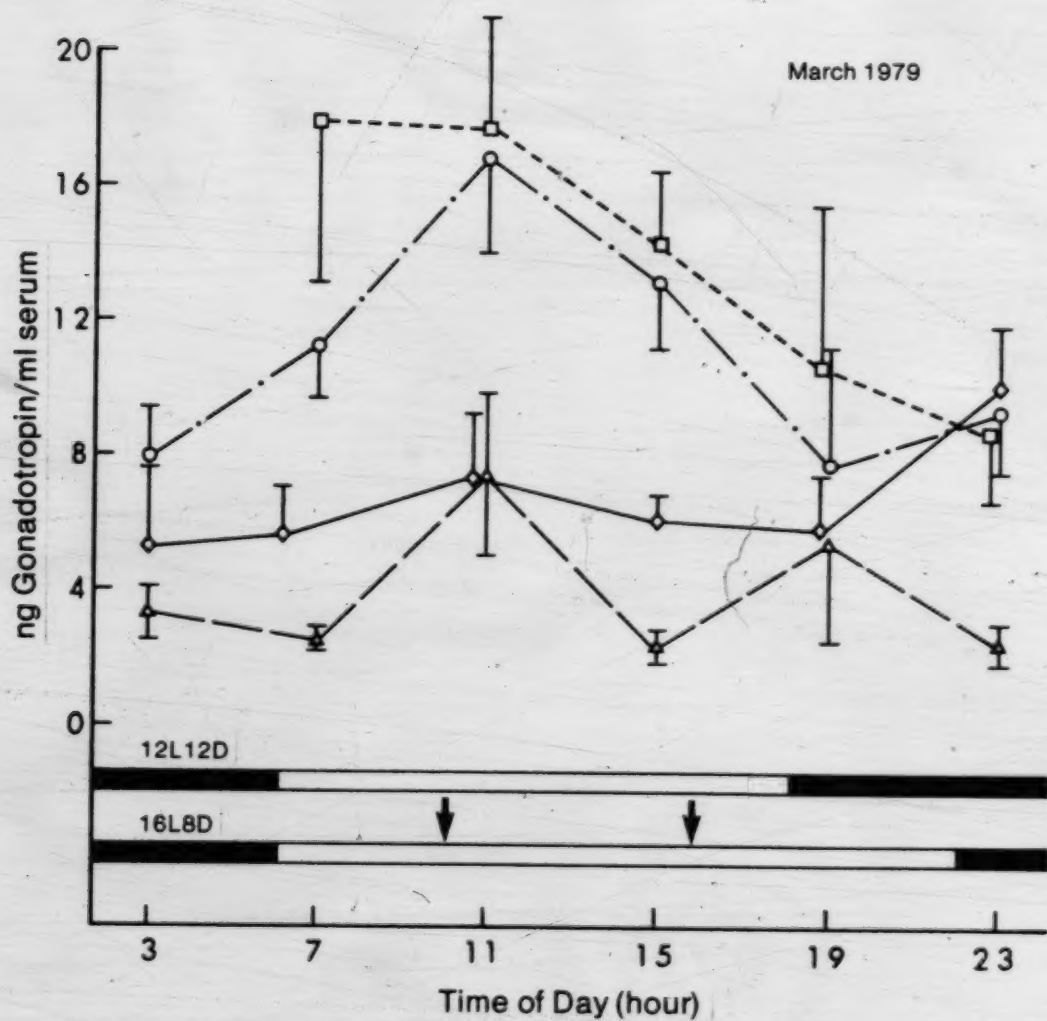
16L8D/20°C

Day 6-8  $\triangle$ — $\triangle$

Day 5-7  $\diamond$ — $\diamond$

Day 11-13  $\circ$ — $\circ$

Day 30-32  $\square$ — $\square$



a peak ( $p < 0.05$ ) in serum GTH levels was found at 1100h (Figure 1.7); in fish held under this regime for an additional 30 to 32 days, peaks ( $p < 0.05$ ) were detected at 0700h and 1900h (Figure 1.8). No significant fluctuations in serum GTH levels were found in the groups held under  $16L8D/20 \pm 1^{\circ}C$  for 5 to 7 days (Figure 1.7) or 30 to 32 days (Figures 1.7, 1.8). A peak ( $p < 0.05$ ) at 1100h was found in the group subjected to  $16L8D/20 \pm 1^{\circ}C$  for 11 to 13 days (Figure 1.7).

Pituitary GTH levels were determined for all the fish in the long term stability experiments done in March, 1979 and no significant fluctuations throughout the day were found in any group (Table 1.2).

Mean serum and pituitary GTH levels found in fish in the long term stability experiments in March, 1979 are shown in Figure 1.9. The mean serum GTH level of the group held under  $12L12D/12 \pm 1^{\circ}C$  for 6 to 8 days was lower ( $p < 0.05$ ) than the mean levels of the other three groups. Also, the mean serum GTH level of the group subjected to  $16L8D/20 \pm 1^{\circ}C$  for 5 to 7 days was lower ( $p < 0.05$ ) than the mean levels found in the groups held under  $16L8D/20 \pm 1^{\circ}C$  for 11 to 13 days and 30 to 32 days. The mean pituitary GTH level of the group subjected to  $16L8D/20 \pm 1^{\circ}C$  for 5 to 7 days was lower ( $p < 0.05$ ) than the levels in fish subjected to this regime for 11 to 13 or 30 to 32 days.

The mean GSI values and the cellular composition of ovaries of fish in the long term stability experiments in March, 1979 are shown in Table 1.3. The mean GSI of the group held under  $12L12D/12 \pm 1^{\circ}C$  for 6 to 8 days was lower ( $p < 0.05$ ) than the mean GSI values of fish held under  $16L8D/20 \pm 1^{\circ}C$  for 5 to 7 or 11 to 13 days. Also, the mean GSI of the group held under  $16L8D/20 \pm 1^{\circ}C$  for 30 to 32 days was lower



Figure 1.8. Serum GTH levels (mean  $\pm$  SE) on days 30 to 32 in fish subjected to 12L12D/12 °C or 16L8D/20 °C in March, 1981.

Numbers of fish sampled are shown beside each mean. See caption of Figure 1.1 for more details.



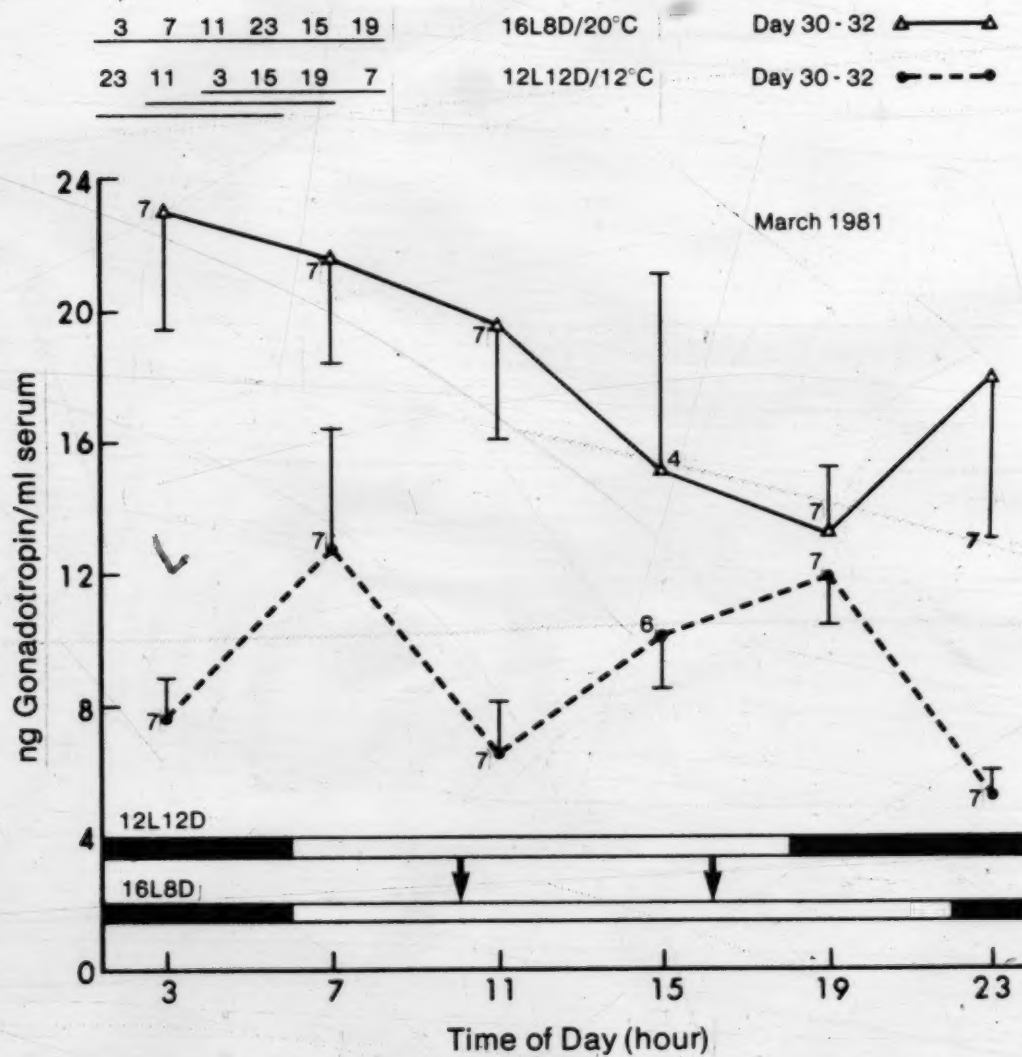


Table 1.2: Pituitary gonadotropin hormone levels expressed as ug gonadotropin/mg of pituitary ( $\bar{x} \pm SE$ ) in fish in the long term stability experiment in March, 1979.

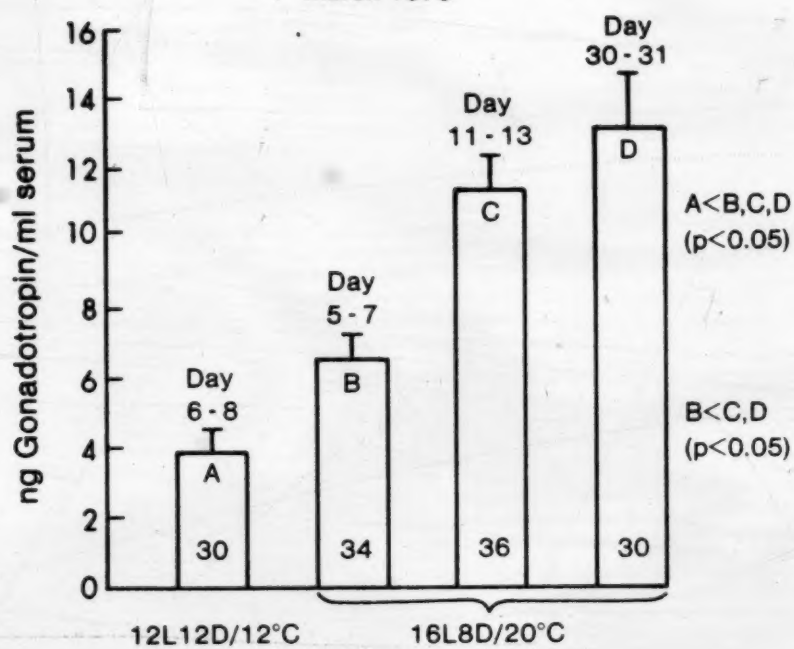
| Experimental group | Time of day         |           |           |           |                    |
|--------------------|---------------------|-----------|-----------|-----------|--------------------|
|                    | 0700h               | 1100h     | 1500h     | 1900h     | 2300h 0300h        |
| 12L12D/12° C       |                     |           |           |           |                    |
| Day 6 to 8         | 70.8±19.4<br>n = 7* | 38.7±6.7  | 42.1±9.4  | 44.7±5.9  | 31.0±6.5 51.7±8.6  |
| 16L8D/20° C        |                     |           |           |           |                    |
| Day 5 to 7         | 42.8±7.9            | 36.5±5.8  | 42.8±9.8  | 51.5±10.2 | 57.8±8.6 37.9±8.5  |
| Day 11 to 13       | 57.7±8.5            | 64.6±10.9 | 52.4±12.7 | 47.1±11.4 | 67.9±12.6 54.3±7.4 |
| Day 30 to 32       | 65.6±6.4            | 57.4±15.6 | 66.1±13.7 | 63.9±16.9 | 58.7±7.2 -         |

\* The average number of fish sampled in an experimental group at each time is 7.

Figure 1.9. Mean serum and pituitary GTH levels (mean  $\pm$  SE) in fish in the long term acclimation experiment in March, 1979.

Numbers of fish sampled are shown at the bottom of the histogram bars. Significant differences by Student's t-test ( $p < 0.05$ ) are indicated at the right of the histograms.

March 1979



serum gonadotropin    pituitary gonadotropin

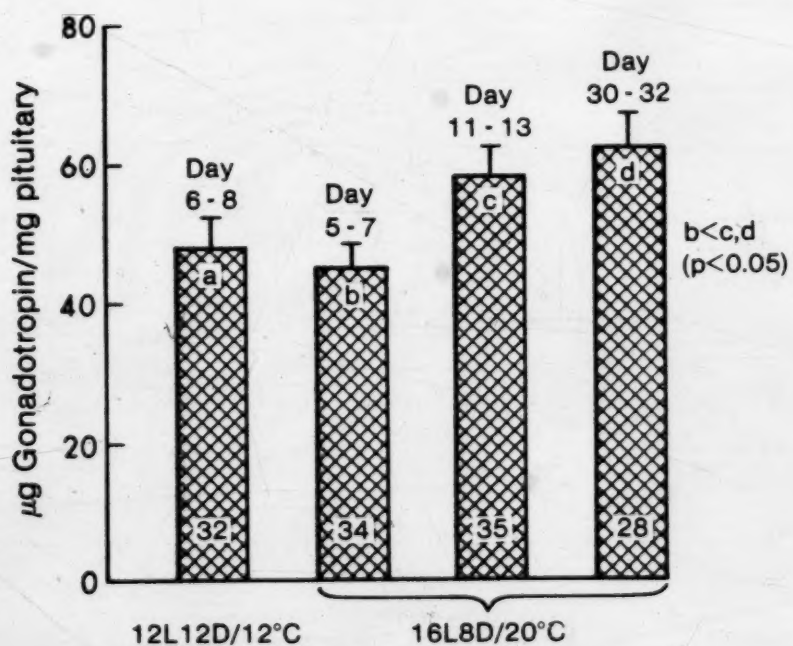




Table 1.3: Cellular composition of ovaries of fish in the long term stability experiments in March, 1979.

| Experimental Group                  | GSI<br>( $\bar{x} \pm SE$ ) | Perinucleolus Stage | Yolk Vesicle Stage | 1 <sup>0</sup> and 2 <sup>0</sup> Yolk Stage | 3 <sup>0</sup> Yolk Stage | Number of severely atretic ovaries (% of total number in a group) |
|-------------------------------------|-----------------------------|---------------------|--------------------|----------------------------------------------|---------------------------|-------------------------------------------------------------------|
| 12L12D/12 <sup>0</sup> C<br>Day 6-8 | 8.5±0.9<br>n=30             | 7.7±2.4<br>n=17     | 1.2±0.4            | 3.4±0.4 <sup>a</sup>                         | 1.6±0.2 <sup>e</sup>      | 0                                                                 |
| 16L8D/20 <sup>0</sup> C<br>Day 5-7  | 11.3±0.9<br>n=34            | 5.9±1.6<br>n=34     | 1.0±0.3            | 2.6±0.3 <sup>b</sup>                         | 2.1±0.2                   | 3                                                                 |
| Day 11-13                           | 12.5±0.8<br>n=36            | 5.9±1.6<br>n=35     | 0.9±0.3            | 2.1±0.3 <sup>c</sup>                         | 2.2±0.1 <sup>f</sup>      | 0                                                                 |
| Day 30-32                           | 9.9±0.9<br>n=30             | 11.7±3.8<br>n=29    | 1.7±0.6            | 0.9±0.2 <sup>d</sup>                         | 2.1±0.2                   | 34*                                                               |

d &lt; a,b,c (p &lt; 0.05); c &lt; a (p &lt; 0.05); e &lt; f (p &lt; 0.05)

\*greatest proportion of severely atretic ovaries found in this group (p &lt; 0.01)

( $p < 0.05$ ) than the mean GSI values of the group held under this regime for 11 to 13 days. Histological examination showed that ovaries of fish subjected to  $16L8D/20 \pm 1^{\circ} C$  for 30 to 32 days contained fewer oocytes in the  $1^{\circ}$  and  $2^{\circ}$  yolk stage than ovaries of fish from the other three groups. Also, ovaries from fish held under  $16L8D/20 \pm 1^{\circ} C$  for 11 to 13 days had fewer oocytes in the  $1^{\circ}$  and  $2^{\circ}$  yolk stage and more oocytes in the  $3^{\circ}$  yolk stage ( $p < 0.05$ ) than ovaries of fish held under  $12L12D/12 \pm 1^{\circ}$  for 6 to 8 days. The greatest proportion ( $p < 0.01$ ) of severely atretic ovaries was found in the group held under  $16L8D/20 \pm 1^{\circ} C$  for 30 to 32 days.

The mean GSI values and the cellular composition of ovaries of fish in the long term acclimation experiment in March, 1981 are shown in Table 1.4. The mean GSI's of the two experimental groups were not significantly different. However, the ovaries of fish held under the  $16L8D/20 \pm 1^{\circ} C$  regime for 30 to 32 days had more ( $p < 0.05$ ) oocytes in the perinucleolus stage and the yolk vesicle stage than ovaries of fish kept for 30 to 32 days under the  $12L12D/12 \pm 1^{\circ} C$  regime. Furthermore, a greater ( $p < 0.05$ ) proportion of severely atretic ovaries were found in the former group. A normal ovary of a mature fish subjected to  $16L8D/20 \pm 1^{\circ} C$  for 11 to 13 days in March is represented in Figure 1.10.1. A severely atretic ovary of a fish subjected to this regime for 30 to 32 days is represented in Figure 1.10.2.

## II. Short term stability experiments

The serum GTH levels in fish subjected to  $16L8D/20 \pm 1^{\circ} C$  for 5 to 7 days in December are shown in Figure 1.11. The GTH levels at 1100h were higher ( $p < 0.05$ ) than the levels at 1900h on each sampling day.

Table 1.4: Cellular composition of ovaries of fish in the long term stability experiments in March, 1981.

| Experimental Group                    | GSI<br>( $\bar{x} \pm SE$ ) | Number of cells/mm <sup>2</sup> of ovary ( $\bar{x} \pm SE$ ) |                    |                                              |                           | Number of severely atretic ovaries<br>(% of total number in a group) |
|---------------------------------------|-----------------------------|---------------------------------------------------------------|--------------------|----------------------------------------------|---------------------------|----------------------------------------------------------------------|
|                                       |                             | Perinucleolus Stage                                           | Yolk Vesicle Stage | 1 <sup>0</sup> and 2 <sup>0</sup> Yolk Stage | 3 <sup>0</sup> Yolk Stage |                                                                      |
| 12L12D/12 <sup>0</sup> C<br>Day 30-32 | 7.7±0.6<br>n=33             | 34.0±5.4<br>n=33                                              | 2.9±0.4            | 15.9±1.4                                     | 3.3±0.6                   | 18                                                                   |
| 16L8D/20 <sup>0</sup> C<br>Day 30-32  | 7.5±0.8<br>n=32             | 70.1±13.7*<br>n=30                                            | 6.6±1.2*           | 13.2±1.9                                     | 4.1±0.7                   | 52*                                                                  |

\* (greater than in the 12L12D/12<sup>0</sup> C group,  $p < 0.05$ )

Figure 1.10. Ovaries in fish in the latter stages of ovarian recrudescence (March) subjected to the 16L8D/20<sup>0</sup> C regime (X25).

1) Normal ovary                      2) Severely atretic ovary

p = oocyte in the perinucleolus stage

s = oocyte in the 1<sup>0</sup> and 2<sup>0</sup> yolk stage

t = oocyte in the 3<sup>0</sup> yolk stage

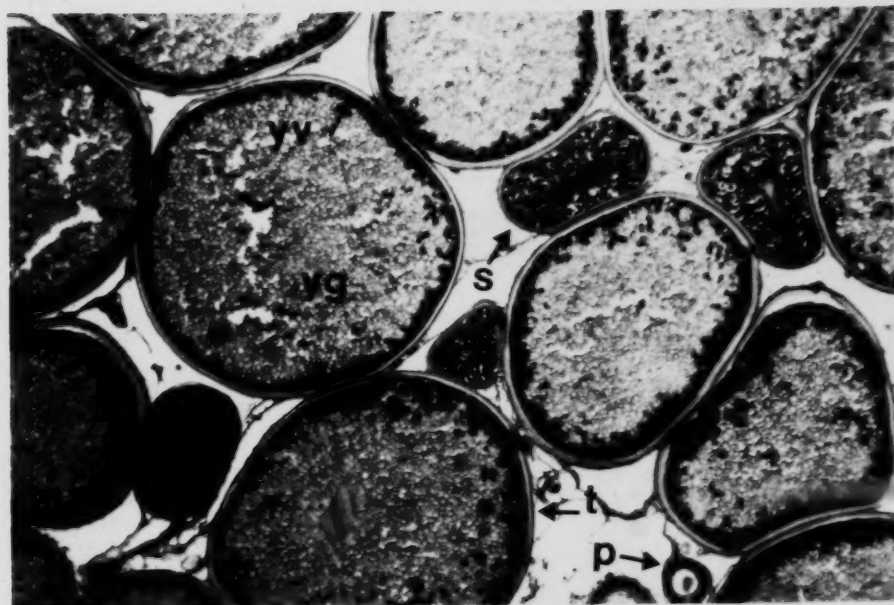
a = atretic oocyte

yg = yolk globules

yv = yolk vesicles



1)



2)

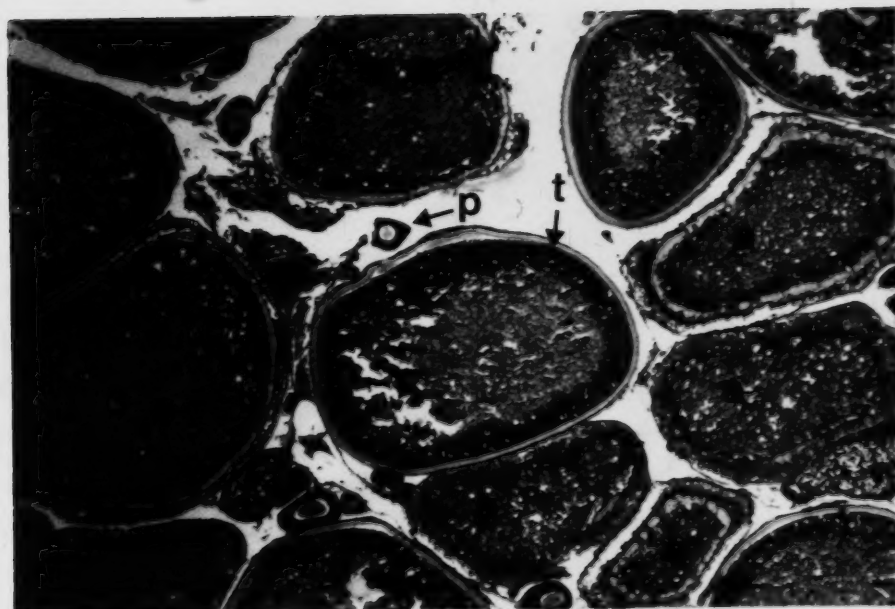
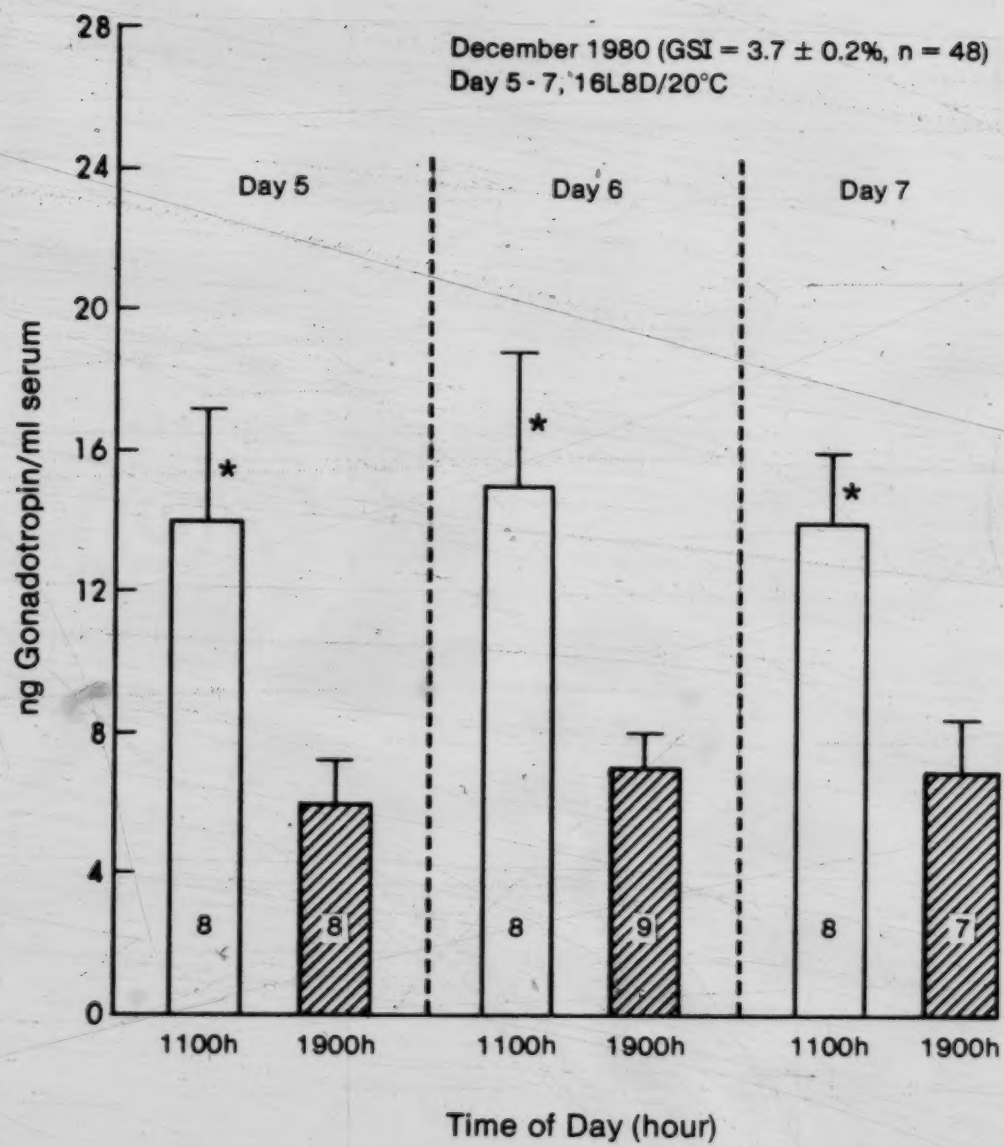


Figure 1.11. Serum GTH levels (mean  $\pm$  SE) on days 5, 6 and 7 in fish subjected to the 16L8D/20 °C regime in December, 1980.

Numbers of fish sampled in each group are shown at the bottom of the histogram bars. The means at 1100h are greater (Student's t-test,  $p < 0.05$ ) than the means at 1900h on each sampling day.



In contrast, the 1100h values on the three days, and the 1900h values on the three days, were not significantly different from each other.

### III. Outdoor experiments

Daily water temperatures, and times of sunrise and sunset during the acclimation periods in September, April and May are presented in Table 1.5. The serum GTH levels, the times of sunrise and sunset, and the temperatures throughout the twelfth day of the outdoor regime are shown in Figures 1.12, 1.13 and 1.14.

In September (Figure 1.12), the serum GTH levels throughout the day were relatively low (about 2 ng/ml of serum) and no significant fluctuations were detected. In April (Figure 1.13), the GTH levels throughout most of the day were similar but a significant peak ( $p < 0.05$ ) was detected at 1500h. One fish in the sample taken at 0300h was ovulated and was rejected from the experiment; the serum GTH level determined in this fish was ten times larger than the average GTH level in the fish sampled at 0300h. Relatively variable GTH levels and no significant fluctuations throughout the day were found in May (Figure 1.14). During the 24 hour sampling period, 22% of the fish ovulated; all ovulated fish were included in the experiment.

### DISCUSSION

The daily variations in serum and, in some experiments, pituitary GTH levels were determined in female goldfish subjected to either the 12L12D/12<sup>0</sup> C or the 16L8D/20<sup>0</sup> C regime for various lengths of time in the laboratory, or to an outdoor pond regime, at several times of the year



Table 1.5: Temperatures, and times of sunrise and sunset during the outdoor experiments.

| SEPTEMBER, 1979 |              |              |                          |        | APRIL, 1981 |              |              |                          |        |
|-----------------|--------------|--------------|--------------------------|--------|-------------|--------------|--------------|--------------------------|--------|
| date            | time         | T °C         | sunrise<br>(time of day) | sunset | date        | time         | T °C         | sunrise<br>(time of day) | sunset |
| Sept 13         | 1020<br>1640 | 14.0<br>14.5 | 0604                     | 1855   | Apr 21      | 1032<br>1605 | 12.0<br>13.0 | 0520                     | 1945   |
| Sept 14         | 1020<br>1700 | 14.5<br>16.0 | 0606                     | 1853   | Apr 22      | 1000<br>1600 | 12.0<br>14.0 | 0518                     | 1947   |
| Sept 15         | 1030<br>1620 | 18.0<br>18.0 | 0608                     | 1850   | Apr 23      | 1005<br>1610 | 13.0<br>15.5 | 0515                     | 1949   |
| Sept 16         | 1015<br>1700 | 16.0<br>-    | 0610                     | 1848   | Apr 24      | 1010<br>1600 | 13.0<br>12.0 | 0513                     | 1951   |
| Sept 17         | 1045<br>1700 | 13.0<br>15.0 | 0611                     | 1846   | Apr 25      | 1010<br>-    | 11.0<br>-    | 0511                     | 1953   |
| Sept 18         | 1010<br>1640 | 14.0<br>15.0 | 0613                     | 1843   | Apr 26      | 1700<br>-    | 11.5<br>-    | 0509                     | 1955   |
| Sept 19         | 1000<br>1600 | 10.5<br>11.5 | 0615                     | 1841   | Apr 27      | 1005<br>1650 | 10.0<br>14.0 | 0507                     | 1956   |
| Sept 20         | 1005<br>1600 | 9.0<br>10.0  | 0617                     | 1838   | Apr 28      | 1000<br>1620 | 11.0<br>14.5 | 0505                     | 1958   |
| Sept 21         | 1030<br>1705 | 8.5<br>9.5   | 0618                     | 1836   | Apr 29      | 1025<br>1615 | 11.0<br>13.0 | 0503                     | 2000   |
| Sept 22         | 1050<br>1645 | 10.0<br>10.5 | 0620                     | 1833   | Apr 30      | 1005<br>1610 | 11.0<br>15.0 | 0500                     | 2002   |
| Sept 23         | 1040<br>1700 | 9.0<br>10.0  | 0622                     | 1831   | May 1       | 1030<br>1600 | 12.5<br>14.0 | 0458                     | 2004   |
| Sept 24         | sampling     | -            | 0624                     | 1828   | May 2       | sampling     | -            | 0456                     | 2005   |

| MAY, 1981 |              |              |                          |        |        |              |              |                          |        |
|-----------|--------------|--------------|--------------------------|--------|--------|--------------|--------------|--------------------------|--------|
| date      | time         | T °C         | sunrise<br>(time of day) | sunset | date   | time         | T °C         | sunrise<br>(time of day) | sunset |
| May 3     | 1600         | 12.0         | 0454                     | 2007   | May 11 | 1045<br>1605 | 13.0<br>15.5 | 0439                     | 2021   |
| May 4     | 1005<br>1635 | 10.0<br>13.0 | 0452                     | 2009   | May 12 | 1015<br>1630 | 14.0<br>17.0 | 0438                     | 2023   |
| May 5     | 1100<br>1610 | 13.0<br>12.5 | 0450                     | 2011   | May 13 | 1010<br>1610 | 14.0<br>16.5 | 0436                     | 2025   |
| May 6     | 1045<br>1625 | 12.5<br>10.0 | 0449                     | 2013   | May 14 | 1010<br>1610 | 14.0<br>14.5 | 0434                     | 2026   |
| May 7     | 1015<br>1630 | 10.5<br>14.0 | 0447                     | 2014   | May 15 | 1050<br>1700 | 11.0<br>12.5 | 0433                     | 2028   |
| May 8     | 1020<br>1630 | 11.4<br>14.0 | 0445                     | 2016   | May 16 | sampling     | -            | 0431                     | 2030   |
| May 9     | 1015<br>1605 | 10.5<br>11.5 | 0443                     | 2018   |        |              |              |                          |        |
| May 10    | 1055<br>1715 | 11.5<br>12.5 | 0441                     | 2019   |        |              |              |                          |        |

Figure 1.12. Serum GTH levels (mean  $\pm$  SE, n) and temperature throughout day 12 of the outdoor pond regime in September, 1979.

Times of sunrise and sunset, and the results of Duncan's multiple range test are shown at the top of the figure. Times of feeding (indicated by arrows) are shown at the bottom.

11 19 15 7 3 23

September 1979, Day 12  
(GSI =  $1.65 \pm 0.1\%$ , n = 51)

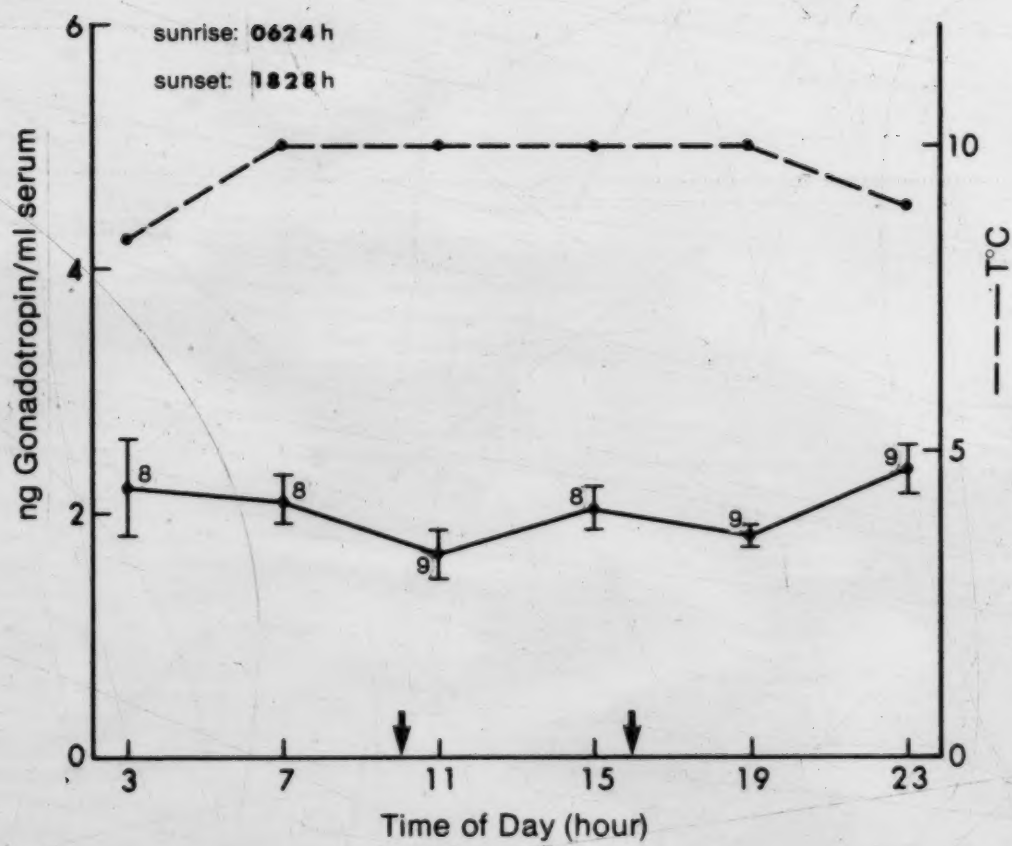


Figure 1.13. Serum GTH levels (mean  $\pm$  SE, n) and temperature throughout day 12 of the outdoor pond regime in April, 1981.

The results of Duncan's multiple range test ( $p < 0.05$ ) are shown at the top of the figure. Times of sunrise and sunset, and feeding are shown at the bottom.



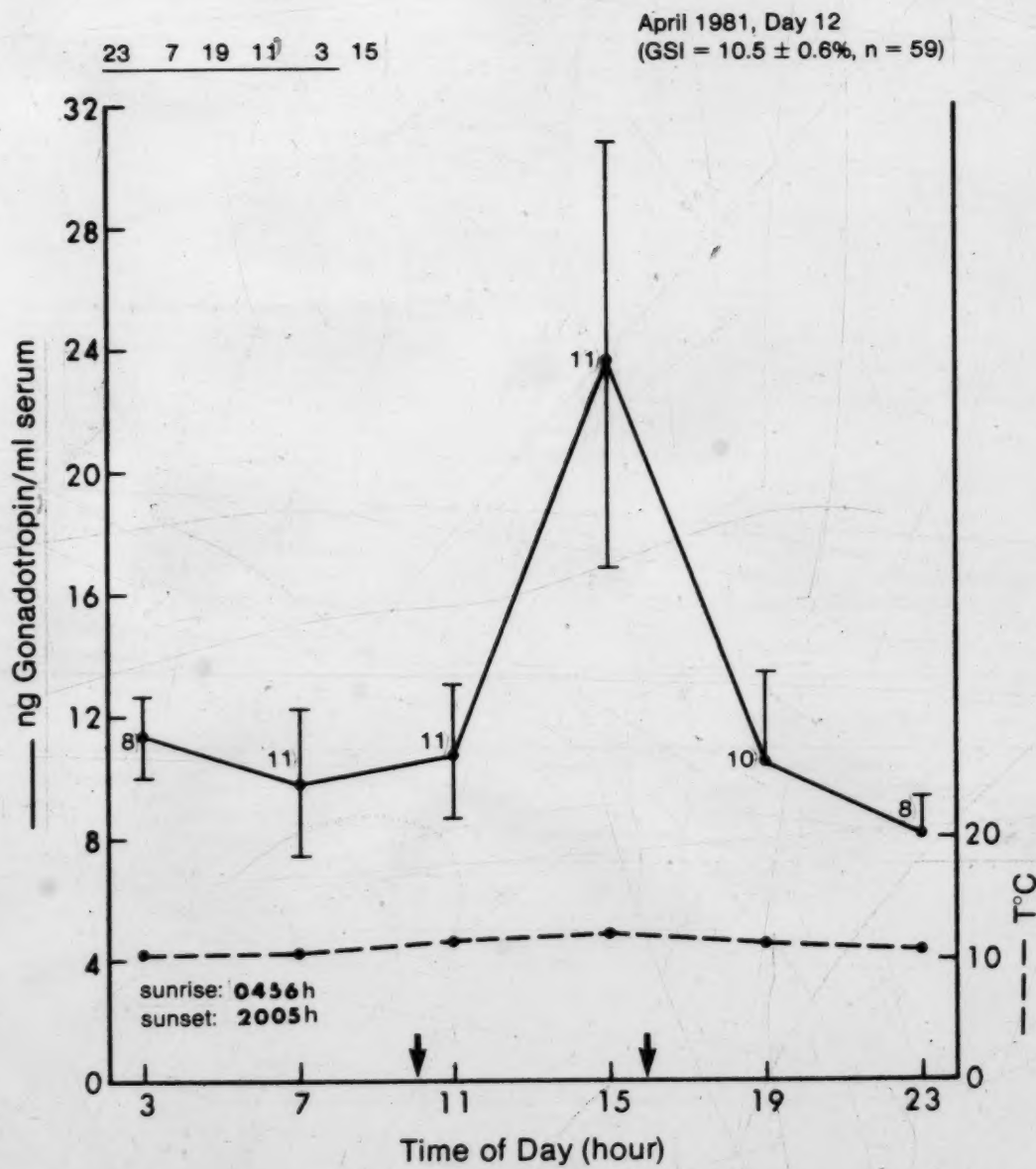
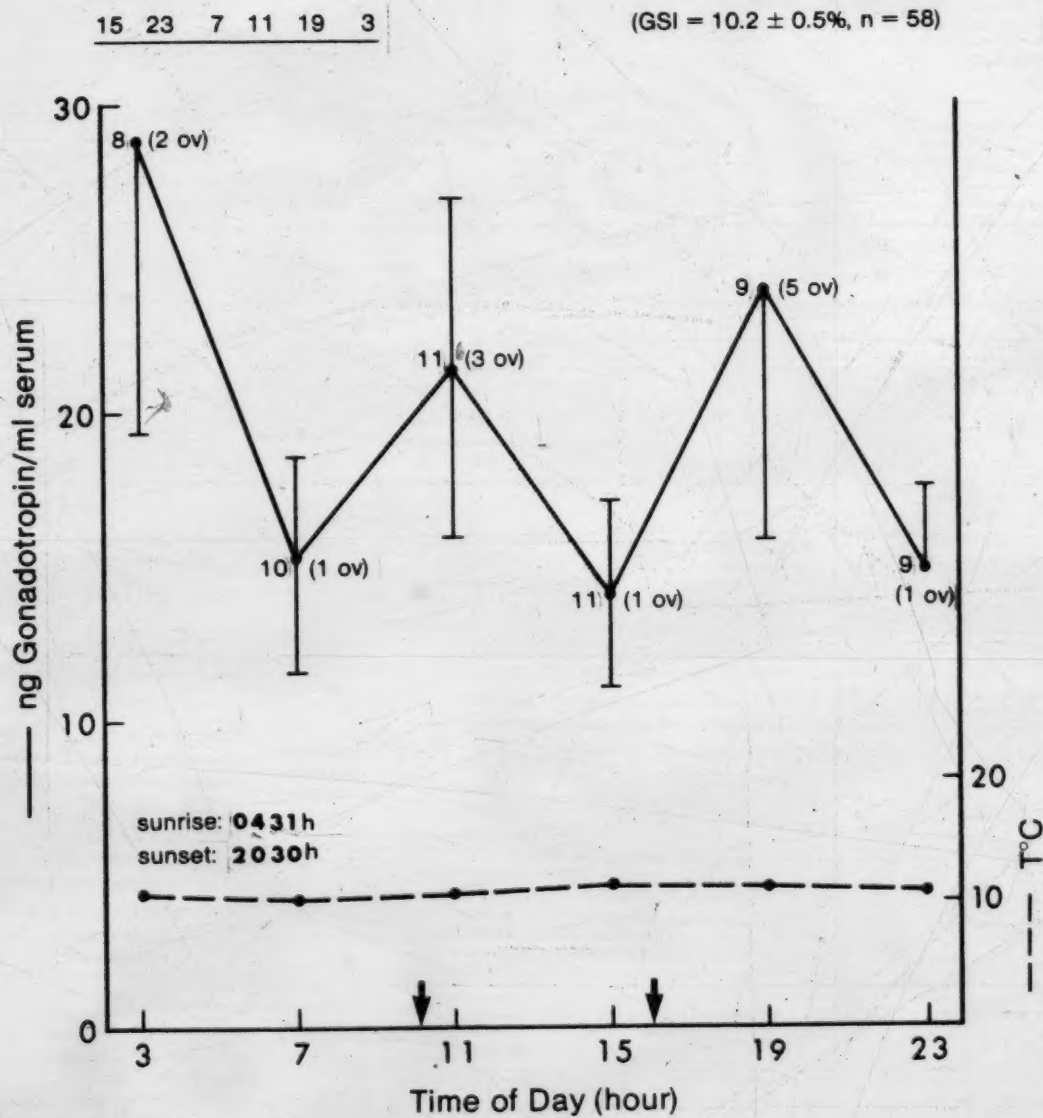


Figure 1.14. Serum GTH levels (mean  $\pm$  SE, n) and temperature throughout day 12 of the outdoor pond regime in May, 1981.

See caption of Figure 1.13 for more detail.

May 1981, Day 12

(GSI =  $10.2 \pm 0.5\%$ ,  $n = 58$ )

While no fluctuations and low serum GTH levels were found in fish in early stages of ovarian recrudescence subjected to 12L12D/12<sup>0</sup> C for up to 32 days in November, significant fluctuations persisting at least up to 32 days were detected in fish in latter stages of ovarian recrudescence held under this regime in March. A single daily peak in serum GTH levels was found in fish held under the 16L8D/20<sup>0</sup> C regime; the peak was evident on days 5 to 7 in November but it was not detected until days 11 to 13 in March. Uniform high serum GTH levels throughout the day were found in November and March in fish subjected to 16L8D/20<sup>0</sup> C for 30 to 32 days. The serum GTH levels in fish subjected to the outdoor conditions of photoperiod and temperature were similar to levels reported previously for fish held under similar, but constant conditions of photoperiod and temperature in the laboratory. Histological data suggested that oocyte growth tended to be greater in fish that had significant fluctuations in serum GTH levels throughout the day than in fish with constant high or low serum GTH levels. Furthermore, constant high serum GTH levels were correlated with atresia of the oocytes.

The experimental design used in the long and short term stability experiments (see Figure 1.1), and also in experiments discussed in other chapters of this study, is similar to the design used by Hontela and Peter (1978, 1980). Therefore, comparison of data is possible and similarities in the patterns of the daily cycles in GTH levels detected in this and previously reported studies are pointed out. As previously, fish were blood sampled at six different times of the day during three consecutive days, and the values were recombined to construct one 24 hour period. The underlying assumption is that the pattern of fluctuations does not change significantly during the three sampling days.



The short term stability experiment in which fish in early stages of ovarian recrudescence were subjected to 12L12D/12<sup>0</sup> C for 8 days, then to 16L8D/20<sup>0</sup> C for 7 days in December, verified this assumption. This particular acclimation regime was used since data provided by the long term acclimation experiment in November showed that a single relatively large mid-day peak in serum GTH levels becomes established within the first 5 to 7 days of exposure to 16L8D/20<sup>0</sup> C. In December, significant fluctuations in serum GTH levels were found on each of the three sampling days, the values at 1100h always being higher than at 1900h. Furthermore, the mid-day peak GTH levels and the nadir GTH levels did not change significantly from day to day. This suggests that the day to day changes in the patterns of the daily cycles in serum GTH are negligible within a given population, and therefore recombining values from three consecutive days to obtain a daily cycle is acceptable. Some support for this has been also provided by the outdoor pond experiments in which fish were sampled in one 24 hour period. Despite the differences between the laboratory and the outdoor conditions of photoperiod and temperature, the cycles detected in fish subjected to similar regimes in the pond and the laboratory were comparable, although fish were sampled throughout three days in the latter.

The long term stability experiments carried out in November and March provided data about the temporal changes in the patterns of daily GTH cycles in fish held under the 12L12D/12<sup>0</sup> C and the 16L8D/20<sup>0</sup> C regimes. The serum GTH levels were relatively uniform and low throughout the day in fish in early stages of ovarian recrudescence subjected to 12L12D/12<sup>0</sup> C for 8 days, or an additional 32 days in November. However,

subsequent to 8 days under this regime, if fish were exposed to 16L8D/20° C, a single peak was detected 5 hours after the onset of light on days 5 to 7, while the levels remained low throughout the rest of the day. As the exposure to the 16L8D/20° C regime was extended, these low levels seemed to gradually increase so that on days 30 to 32 the mid-day peak was no longer evident and the serum GTH levels were relatively constant and high throughout the entire day. These data confirm and extend data reported by Hontela and Peter (1978) who demonstrated that no fluctuations and low serum GTH levels were found in recrudescing fish subjected to short photoperiod and cold temperature, and a mid-day peak was detected in fish subjected to long photoperiod and warm temperature for 7 to 9 days in January.

In the November long term experiment, significant daily fluctuations in pituitary GTH levels were found in the two groups of fish in early recrudescence exposed to 12L12D/12° C for 6 to 8 days and to 16L8D/20° C for 5 to 7 days. In the former group, the highest GTH levels in both the serum and pituitary were found at 2300h, but a positive correlation of the two parameters was not found. Similarly, although the patterns of daily fluctuations in serum and pituitary GTH levels in fish held under 16L8D/20° C for 5 to 7 days was highly suggestive of a negative correlation between the two, the correlation was not significant. Vodcnik *et al.* (1978) also determined serum and pituitary GTH levels at two times of the day in the goldfish, and did not find a positive or negative correlation.

Although a daily cycle in the release of GTH from the pituitary can be assumed to underlie the daily cycle in blood levels of GTH, when

the hourly secretion rate of GTH is calculated from the metabolic clearance rate (Cook and Peter, 1980a) and the serum GTH levels, it appears that the amount of GTH released hourly is only a small proportion of the amount of GTH stored in the pituitary. How synthesis and degradation of GTH in the pituitary relate to the secretion rate is not known. Mean pituitary and serum GTH levels were increased in fish exposed to 16L8D/20<sup>0</sup> C for 5 to 7 days compared to the levels found in fish held under 12L12D/12<sup>0</sup> C. While the mean serum GTH levels increased further on days 11 to 13 and remained high on days 30 to 32, the mean pituitary GTH levels on days 11 to 13 and 30 to 32 were similar to the levels found in fish subjected to 12L12D/12<sup>0</sup> C. Possibly, the pituitary GTH levels decreased when exposure to the 16L8D/20<sup>0</sup> C regime was extended because the release of GTH exceeded synthesis, although further investigations are required to test this hypothesis. Nevertheless, since the patterns of serum GTH levels changed independent of the changes in pituitary content, it seems that, in the present case, GTH synthesis is not a determining factor in the daily cycle of GTH release.

The long term stability experiments carried out in March provided data permitting a comparison of the long term effects of the 12L12D/12<sup>0</sup> C and 16L8D/20<sup>0</sup> C regimes on GTH levels in fish in early recrudescence (November) and in fish in the latter stages of recrudescence (March). Two daily peaks in serum GTH levels were found in fish subjected to the 12L12D/12<sup>0</sup> C regime in March, one around the onset of light, and the other 13 hours later. Although only one of the peaks was clearly defined after the first 8 days of exposure to the 12L12D/12<sup>0</sup> C regime, the cycle with two peaks persisted for at least 32 days. A similar daily cycle has been reported by Hontela and Peter (1978) in fish subjected

to short photoperiod and cold temperature for 7 to 9 days in spring; the first peak was found at the onset of light, the second 12 hours later. A single mid-day peak in serum GTH levels was found in fish exposed to 16L8D/20<sup>0</sup> C for 11 to 13 days in March in the present study; no significant fluctuations were found after 5 to 7 days, in contrast to the November experiment. Interestingly, a mid-day peak was found by Hontela and Peter (1978, 1980) in mature females exposed to 16L8D/20<sup>0</sup> C for 7 to 9 days in March and April. At present, it can only be postulated that in fish in latter stages of ovarian recrudescence an exposure to 16L8D/20<sup>0</sup> C of at least 7 to 9 days is required before the daily cycle in serum GTH levels is established. No significant daily fluctuations in serum GTH levels were found after exposure to 16L8D/20<sup>0</sup> C for 30 to 32 days; the GTH levels were high and relatively constant throughout the day, although the highest levels were still found in the early portion of the photophase. The mean serum GTH levels were greater in the group held under 16L8D/20<sup>0</sup> C for 5 to 7 days than in the group held under the initial acclimation regime, and further increases in the serum GTH levels occurred at 11 to 13 days and 30 to 32 days. The pituitary GTH levels did not fluctuate throughout the day in any of the groups in the March long term experiment. The mean pituitary GTH levels in the fish held under the 16L8D/20<sup>0</sup> C for 5 to 7 days were similar to those exposed to the initial acclimation regime, but were significantly less than in fish exposed to 16L8D/20<sup>0</sup> C for 11 to 13 or 30 to 32 days. The data concerning the daily cycles or the means levels in serum and pituitary GTH levels in March show, similar to the November experiment, that serum GTH levels may change independent of the pituitary GTH levels.



The present study demonstrates that there are differences in the responses of female goldfish to exposure to 12L12D/12<sup>0</sup> C and 16L8D/20<sup>0</sup> C regimes in November and March in terms of the patterns of the daily cycles in serum GTH levels. While relatively constant and low serum GTH levels were found in fish in early recrudescence subjected to 12L12D/12<sup>0</sup> C in November for up to 32 days, a daily cycle with two peaks in serum GTH levels which persisted for at least 32 days was detected under this regime in fish in latter stages of recrudescence in March. A daily cycle with one peak 5 hours after the onset of light was detected in fish held under 16L8D/20<sup>0</sup> C regime in both November and March. However, while this pattern could be detected on days 5 to 7 in November, 11 to 13 days were required in March. The difference in the gonadal condition of fish undergoing early ovarian recrudescence and mature fish with ovaries containing some oocytes that completed vitellogenesis may be an important factor causing the observed differences in responses of the fish to the 12L12D/12<sup>0</sup> C and 16L8D/20<sup>0</sup> C regimes in November and March. Seasonal changes in the blood levels of sex steroids (Schreck and Hopwood, 1974; Y. Nagahama, personal communication) and the ovarian feedback effect on the hypothalamo-hypophysial axis may influence the responsiveness of the axis to environmental cues at various times of the year. Variations throughout the year in the effect of castration (Billard *et al.*, 1976, 1977; Billard, 1978) and steroid treatment (Billard, 1978) on GTH secretion in rainbow trout have been reported. Seasonal differences in the effects of pinealectomy, blinding and melatonin administration on daily cycles in serum GTH levels in goldfish were also observed in the present study (Chapters 2, 3 and 4).

The present study provides data which allow, for the first time, a comparison of the daily cycles in serum GTH levels in fish subjected to laboratory and outdoor pond conditions. Fish were fed at 1000h and 1600h in all the pond experiments, in an attempt to control this environmental variable. The effect of feeding on daily GTH cycles is investigated in Chapter 2. Fish subjected to the outdoor pond conditions in September had regressed gonads and the serum GTH levels found in these fish were constant and low throughout the day. This is consistent with previously reported data which showed that small or no fluctuations in serum GTH levels are found in regressed fish exposed to various photoperiod and temperature regimes (Vodicnik *et al.*, 1978; Hontela and Peter, 1978, 1980). On the other hand, in the pond experiment in April, a significant peak in serum GTH levels was found 10 hours after sunrise in females in the latter stages of ovarian recrudescence. The temperature throughout this experiment was cold and the photoperiod was gradually increasing; on the day of sampling a 15L9D/11<sup>0</sup> C regime was recorded. The pattern in serum GTH levels was very similar to the one reported for mature fish subjected to the 16L8D/12<sup>0</sup> C regime for 7 to 9 days in March in the laboratory; a single peak in serum GTH levels was detected 12 hours after the onset of light (Hontela and Peter, 1978). In the outdoor experiment in May, several fish (22%) ovulated during the experiment and no significant daily fluctuations were detected, whether or not the serum GTH values of the ovulated fish were included in the experiment. Serum GTH levels increase dramatically during the preovulatory surge of GTH in the goldfish (Stacey *et al.*, 1979a), and the highly variable serum GTH levels found in fish in the May experiment

might be caused by ovulations which were not synchronized within the population. In conclusion, the data provided by the outdoor experiments demonstrated that the cycles in serum GTH in fish kept in an outdoor pond are similar to the cycles found in the laboratory, when similar photoperiod and temperature regimes and sexual conditions are compared. Furthermore, although the effects of warm temperature regimes were not investigated in the present study in an outdoor pond, Breton *et al.* (1972) reported a daily cycle in serum GTH levels in mature female goldfish held under outdoor summer conditions of photoperiod and temperature. The pattern was similar to the one reported by Hontela and Peter (1978, 1980) for mature fish held under a 16L8D/20<sup>0</sup> C regime in March in the laboratory.

The data from the outdoor experiments provide some support for the hypothesis that daily cycles in serum GTH levels are important for ovarian development, since a significant daily fluctuation in serum GTH levels was found in preovulatory mature females kept under natural conditions of photoperiod and temperature. The present study also provided data permitting a correlation between the patterns of the daily cycles in serum GTH levels detected in fish subjected to laboratory photoperiod and temperature regimes, and ovarian condition as determined by histology. In general, a significant daily fluctuation in serum GTH levels was coincident with fish having ovaries with few atretic oocytes and with oocytes that seemed to be growing more rapidly than in fish having constant and low serum GTH levels throughout the day. In addition, constant high serum GTH levels throughout the day correlated with atretic oocytes. The long term stability experiment carried out with fish

undergoing early stages of ovarian recrudescence in November illustrates these points. Ovarian development, as indicated by a larger number of oocytes in the 1<sup>0</sup> and 2<sup>0</sup> yolk stage, was more advanced in fish subjected to 16L8D/20<sup>0</sup> C for 11 to 13 days, than in fish held under 12L12D/12<sup>0</sup> C for 8 or 32 days. A similar but statistically nonsignificant trend was observed in the group held under 16L8D/20<sup>0</sup> C for 5 to 7 days. Fluctuations in serum GTH levels were found only in the two groups exposed to 16L8D/20<sup>0</sup> C, although only in the group held under this regime for 5 to 7 days were they significant. Furthermore, the greatest proportion of atretic ovaries was found in the group held under the 16L8D/20<sup>0</sup> C regime for 32 days, a group in which serum GTH levels were high and constant throughout the day. Similar trends were also observed in the long term stability experiment carried out with females in the latter stages of ovarian recrudescence in March. Constant high levels in serum GTH were correlated with a high proportion of severely atretic ovaries in the groups held under 16L8D/20<sup>0</sup> C for 32 days in March, 1979 and 1981. With the exception of the group kept under 16L8D/20<sup>0</sup> C for 5 to 7 days, the groups exposed to 16L8D/20<sup>0</sup> C for 11 to 13 days, and 12L12D/12<sup>0</sup> C for 6 to 8 days or 30 to 32 days had very few atretic oocytes and ovaries that were apparently actively undergoing vitellogenesis coincident with significant fluctuations in serum GTH levels. The present study suggests that high GTH levels in the blood stimulate ovarian development only if the high levels are imposed during restricted portions of the day, and not over the entire 24 hour period. Although Gillet *et al.* (1978) reported ovarian regression in goldfish that had high serum GTH levels, studies in which GTH levels were determined once a day showed that increased blood GTH levels are generally associated with a stimulation of



ovarian development (for review, see Peter, 1981). However, it is not known whether the blood GTH levels in these experiments were constantly high throughout the 24 hour period, because multiple sampling was not carried out and the time of sampling was usually not specified.

Reports of stimulation of gonadal maturation by GTH administered by pellet implantations in the goldfish (Khoo, 1980) and pink salmon (MacKinnon and Donaldson, 1978) challenges the postulation that fluctuating serum GTH levels promote gonadal development, since it may be assumed that GTH was released from the capsule at a steady, and possibly relatively high, rate. However, blood GTH levels were not determined in these studies and the contribution of endogenous GTH secretion is not known in these circumstances. Similar experiments carried out with hypophysectomized fish implanted with GTH capsules could be designed to test further the hypothesis that steady and high-serum GTH levels are detrimental to the teleost ovary.

Evidence for a physiological role of the daily cycles in blood levels of GTH in stimulation of gonadal development in teleosts is still limited. The responsiveness of the ovary to exogenous GTH has been shown to vary throughout the day in the shiner (de Vlaming and Vodcnik, 1977) and goldfish (Peter *et al.*, 1982); a greater stimulation of ovarian growth was achieved by GTH injections given at one particular time of the day than at other times. In the goldfish acclimated to 16L8D/14<sup>0</sup> C, the time of greatest responsiveness to injected GTH coincided with the time of the daily peak in serum GTH as reported by Hontela and Peter (1978). Similarly, Kuo and Watanabe (1978) demonstrated daily variations in the production of c-AMP in response to GTH or

prostaglandins by ovaries of *Mugil cephalus*. These data suggest that the stimulatory effect of GTH on the ovary does not depend only on the amount of GTH available throughout the day, but also the time of day during which GTH is present. The daily variations in the response of the teleostean gonads to GTH might be related to daily fluctuations in the number and/or activity of the gonadal GTH receptors. The desensitizing effect of constant and high serum GTH levels on the ovary in mammals is thought to be the result of down regulation of ovarian GTH receptors (see Introduction); data provided by the present study suggest that this may also occur in the goldfish. Although ovarian GTH receptors were characterized and/or manipulated in representatives of all vertebrate classes (e.g. Chan *et al.*, 1981; Tsutsui and Ishii, 1978; Licht *et al.*, 1977; Kubokawa and Ishii, 1980), such investigations have not yet been carried out in teleosts.

The possibility remains that the atresia of oocytes in the presence of high and constant blood GTH levels may not be directly caused by the pattern of GTH levels, but rather, by other factors associated with the long term exposure to the 16L8D/20<sup>0</sup> C regime. It has been reported that temperature influences sex steroid synthesis by the testis of the goldfish (Kime, 1980) and the hepatic catabolism of steroids in rainbow trout and goldfish (Kime and Saksena, 1980). In addition, Wiegand and Peter (1980) reported changes in lipid metabolism in goldfish caused by temperature. Possibly, long exposure to 20<sup>0</sup> C influences steroid or lipid metabolism in such a way as to cause atresia of the oocytes.

The present study demonstrated that predictable daily cycles in serum GTH levels occur in fish subjected to specific sets of photoperiod

and temperature conditions. The cycles are similar in fish kept in the laboratory and in fish subjected to similar outdoor conditions of light and temperature. Furthermore, while significant daily fluctuations in serum GTH levels seem to stimulate ovarian development more than constant low levels in most instances, constant high serum GTH levels were associated with atresia of the oocytes.

## CHAPTER 2: EFFECTS OF ENVIRONMENTAL CUES ON DAILY GONADOTROPIN HORMONE CYCLES

### INTRODUCTION

It is well established that seasonally breeding teleost fishes use various environmental cues to regulate their annual reproductive cycles. Both photoperiod and temperature have been shown to influence gonadal development in many teleosts. Although exceptions are known, most salmonid fishes accelerate gonadal recrudescence under decreasing photoperiod and cold temperatures, while gonadal development in cyprinids is stimulated by increasing photoperiod and warming temperatures (for review, see Htun-Han, 1977; Billard and Breton, 1978; Billard *et al.*, 1978; Peter and Crim, 1979; and Peter, 1981). Extensive work has been carried out on the effects of environmental cues on blood or pituitary GTH levels (for a recent review, see Peter, 1981), but few studies have attempted to determine the effects of such cues on GTH levels throughout 24 hour periods. The few reported studies were concerned with the effects of photoperiod and temperature only (Breton *et al.*, 1972; Vodcnik *et al.*, 1978; Hontela and Peter, 1978, 1980), whereas the effects of other cues such as feeding times have not been considered. The pattern of the daily cycle in serum GTH levels is influenced by photoperiod, temperature, sexual condition, and, as shown in Chapter 1, by the length of the acclimation period to the environmental regime. Although data presented in this manuscript indicate that the patterns of the cycles are predictable in fish subjected to specific sets of environmental conditions in the laboratory and in an outdoor pond, the relative importance of the



various environmental cues in entraining and modulating the pattern of the daily cycles in serum GTH levels is not known.

The light-dark cycle (LD) is a powerful entraining or synchronising stimulus for various daily rhythms, including true circadian rhythms in plants and animals (Bünning, 1973; Menaker and Zimmerman, 1976; Kavaliers, 1979; Wever, 1980; Czeisler *et al.*, 1981), and various daily endocrine rhythms (including those of teleost fishes, for review see Matty, 1978) that may not be truly circadian since they do not persist under constant conditions. It has been established that the preovulatory LH surge in many mammalian species is synchronised to the LD cycle (Colombo *et al.*, 1974; Baldwin and Sawyer, 1979). In mature male Japanese quail kept under a long photoperiod, a peak in the gonadotropic activity of the pituitary is usually detected near the end of the light phase; a shift of the onset of the photoperiod results in a shift of the peak towards the end of the new light phase (Hashigushi *et al.*, 1977b). Stacey *et al.* (1979b) showed in the goldfish that the timing of spontaneous ovulation is synchronised with photoperiod, ovulation occurring in the latter part of the dark phase even under reversed photoperiod. Since ovulation in goldfish is preceded by a surge in blood levels of GTH (Stacey *et al.*, 1979a), these data suggest that the surge can be synchronised by LD. The effects of shifts in the LD cycle on the patterns of the daily cycles in serum GTH levels have not been investigated, however.

Feeding schedules have been shown to entrain some daily endocrine rhythms. Daily fluctuations in plasma corticosterone levels in the rat are synchronised by the LD cycle and feeding times (Fukuda *et al.*, 1977;

Holloway *et al.*, 1980); in constant light conditions (LL or DD), the feeding schedule alone can entrain the rhythm (Krieger, 1974, 1980; Krieger *et al.*, 1977; Morimoto *et al.*, 1977, 1979; for review, see Boulos and Terman, 1980). Daily cycles in serum cortisol and total corticoids levels have been detected in the goldfish (Fryer, 1975; Delahunty *et al.*, 1978b; Peter *et al.*, 1978b), and recently, it has been demonstrated that shifting the timing of the single daily meal will shift the peak in plasma cortisol (Spieler and Noeske, 1981), or modify the pattern of the daily cycle in corticoids levels (Delahunty *et al.*, 1978b). A daily cycle in plasma thyroxine levels detected in rainbow trout (White and Henderson, 1977; Osborne *et al.*, 1978) and goldfish (Spieler and Noeske, 1979) can be modified but not shifted by changes in the times of feeding (Spieler and Noeske, 1981; Eales *et al.*, 1981). Similarly, the pattern of daily fluctuations in liver glycogen, and plasma glucose and lipid levels in the goldfish were influenced by changes in feeding times (Delahunty *et al.*, 1978b). It seems therefore, that a shift of feeding times can either shift or modify the patterns of some daily endocrine and/or metabolic rhythms in teleosts. The effects of feeding times on the daily GTH cycles in teleosts have not been determined and furthermore, the relative importance of feeding times and the LD cycle in entraining the daily rhythms has not been investigated.

Little is known about the effects of temperature in entrainment of daily hormonal cycles in teleosts. The relevance of this question is suggested by the fact that poikilothermic organisms may experience diurnal fluctuations in temperature in their natural environment, and the fact that some fishes, including the goldfish, exhibit diel rhythms

of preferred temperature in free choice experiments in the laboratory (Reynolds *et al.*, 1978a, 1978b; Reynolds and Casterlin, 1978). It has also been reported that the circadian rhythmicity of events such as eclosion and diapause in insects can be entrained solely by daily thermocycles under constant light conditions (Zimmerman *et al.*, 1968; Saunders, 1973).

In the present study, the effects of shifts in the LD cycle and feeding times, and also of a diurnal temperature regime on the daily cycle in serum GTH levels and ovarian development in the goldfish were investigated. In the first series of experiments, it was determined whether a simultaneous shift in the onset of light and the feeding times caused an appropriate shift in the pattern of the GTH cycle. Next, the relative importance of the LD cycle and feeding in entrainment of the GTH cycle was investigated by shifting the LD cycle and the feeding times separately. The effects of sinusoidal diurnal temperature regimes, with the warm phase being imposed either during day or during night, on the GTH cycle were also investigated.

#### MATERIALS AND METHODS

See Materials and Methods of Chapter 1 for descriptions of experimental animals (section I), initial acclimation regime (section II), blood sampling technique (section III), radioimmunoassay for GTH (section IV), histology (section V) and statistical analysis (section VI).

## I. Environmental regimes and sampling schedules

### Light and feeding shift experiment

Following the initial acclimation period, selected fish were transferred into two flow-through 380 L aquaria maintained at  $12L12D/12 \pm 1^{\circ}C$ . Light went on at 0600h and the fish were fed at 1000h and 1600h in the control group. In the experimental group, the onset of light and the feeding times were shifted by 8 hours, so that the light went on at 1400h and the fish were fed at 1800h and 2400h. After 8 days, the experimental regime ( $16L8D/20 \pm 1^{\circ}C$ ) was imposed for 11 to 13 days. Lights were turned on and fish were fed at the same times as during the 12L12D regime (see Figure 2.1). Blood was sampled at the standard sampling times throughout days 11, 12 and 13 of the  $16L8D/20 \pm 1^{\circ}C$  regime in April; GTH levels in the blood and GSI's were determined.

### Light or feeding shift experiments

Following the initial acclimation period, fish were weighed, transferred into three 380 L flow-through aquaria and subjected to  $12L12D/12 \pm 1^{\circ}C$  for 8 days. Light went on at 0600h and fish were fed at 1000h and 1200h in the control group. In the second group ("feeding shift" group), light went on at 0600h and fish were fed at 1600h and 1800h. In the third group ("light shift" group), light went on at 2400h and fish were fed at 1000h and 1200h. Then, the experimental regime ( $16L8D/20 \pm 1^{\circ}C$ ) was imposed for either 5 to 7 or 11 to 13 days. During this period, the light went on at the same time as during the 12L12D regime but the feeding times were changed: the control and the "light shift" groups were fed at 1000h and 1500h, and the "feeding shift" group was fed at 1600h and 2100h (Figure 2.2). Blood GTH levels and GSI's were



Figure 2.1. Serum GTH levels (mean  $\pm$  SE, n) on days 11 to 13 of the 16L8D/20  $\pm$  1  $^{\circ}$ C regime in fish subjected to an 8 hour shift in the onset of light and feeding times, and in fish held under a control regime.

The results of the Duncan multiple range test ( $p < 0.05$ ) are shown at the top of the figure. The feeding times (indicated by arrows) and the experimental photoperiod regimes are shown at the bottom; the black horizontal bars represent the dark phase of the photoperiod, the empty bars represent the light phase.

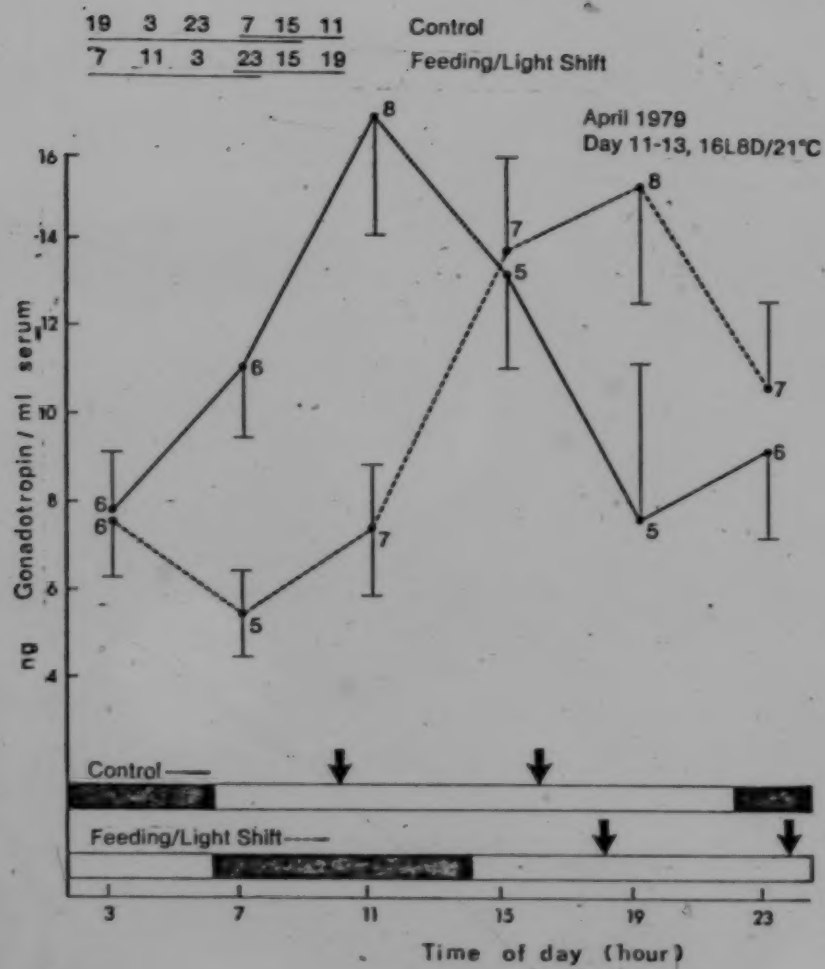
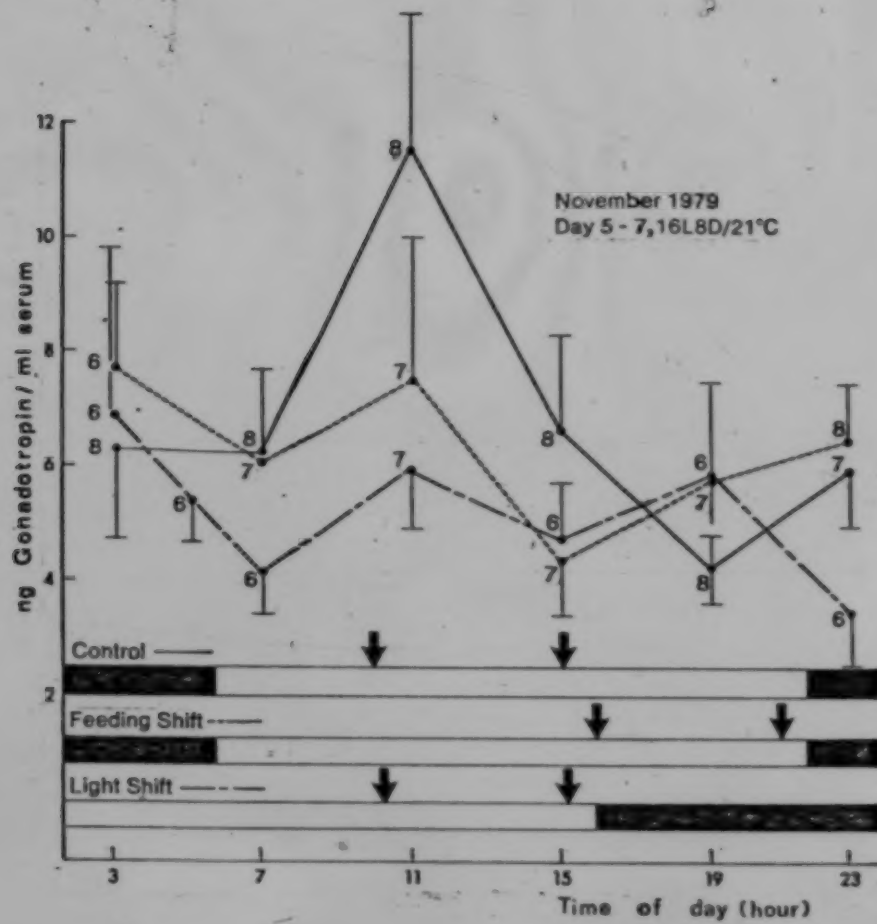


Figure 2.2. Serum GTH levels (mean  $\pm$  SE, n) on days 5 to 7 of the 16L8D/20  $\pm$  1  $^{\circ}$ C regime in November in fish subjected to a 6 hour shift either in the onset of light or in the feeding times, and in fish held under a control regime.

The results of the Duncan multiple range test ( $p < 0.05$ ) are shown at the top of the figure; the feeding times (indicated by arrows) and the photoperiod regimes are shown at the bottom.

|           |           |           |           |           |           |                      |
|-----------|-----------|-----------|-----------|-----------|-----------|----------------------|
| <u>15</u> | <u>23</u> | <u>7</u>  | <u>3</u>  | <u>19</u> | <u>11</u> | Control              |
| <u>15</u> | <u>19</u> | <u>7</u>  | <u>23</u> | <u>11</u> | <u>3</u>  | Feeding Shift        |
| <u>23</u> | <u>7</u>  | <u>15</u> | <u>5</u>  | <u>19</u> | <u>11</u> | <u>3</u> Light Shift |





determined at the standard sampling times throughout days 5, 6 and 7 (November) and days 11, 12 and 13 (April) of the 16L8D/20  $\pm$  1 $^{\circ}$  C regime. Some groups were also sampled at a few additional times (see Results, Figures 2.2 and 2.3). Histological analysis of the ovaries was carried out in April only.

#### Temperature experiment

Following the initial acclimation period, fish were transferred into three 380 L flow-through aquaria and held under 12L12D/12  $^{\circ}$  C for 8 days. The light went on at 0600h and fish were fed at 1000h and 1600h; the onset of light and feeding times remained the same throughout the entire experiment. Subsequently, the photoperiod was extended to 16L8D in all three groups and the experimental temperature regimes (see Figure 2.4) were imposed. In the control group, the temperature was raised from 12 $^{\circ}$  C to 20 $^{\circ}$  C during the first 12 hours of the first day of the 16L8D regime (starting at 0800h) and maintained at 20  $\pm$  1 $^{\circ}$  C for the entire experiment. In the second group ("high day" group), the temperature started to increase at 0800h, 20 $^{\circ}$  C was reached at about 1100h and was maintained until 2000h. At this time, temperature started to decrease, 12 $^{\circ}$  C was attained at about 2300h and maintained until 0800h. In a similar fashion, the temperature was raised to 20 $^{\circ}$  C during night and lowered to 12 $^{\circ}$  C during day in the third group ("low day" group). Fish were sampled at the standard sampling times throughout day 14, 15 and 16 of the experimental regimes in February. Blood GTH levels, GSI's and the histological status of the ovaries were determined.

Figure 2.3. Serum GTH levels (mean  $\pm$  SE, n) on days 11 to 13 of the 16L8D/20  $\pm$  1  $^{\circ}$ C regime in April in fish subjected to a 6 hour shift either in the onset of light or in the feeding time, and in fish held under a control regime.

The results of the Duncan multiple range test ( $p < 0.05$ ) are shown at the top of the figure; the feeding times (indicated by arrows) and the photoperiod regimes are shown at the bottom.

|    |   |    |    |   |    |    |
|----|---|----|----|---|----|----|
| 15 | 7 | 19 | 23 | 3 | 11 |    |
| 19 | 7 | 11 | 15 | 3 | 17 | 23 |
| 15 | 7 | 19 | 3  | 5 | 11 | 23 |

Control  
Feeding Shift  
Light Shift

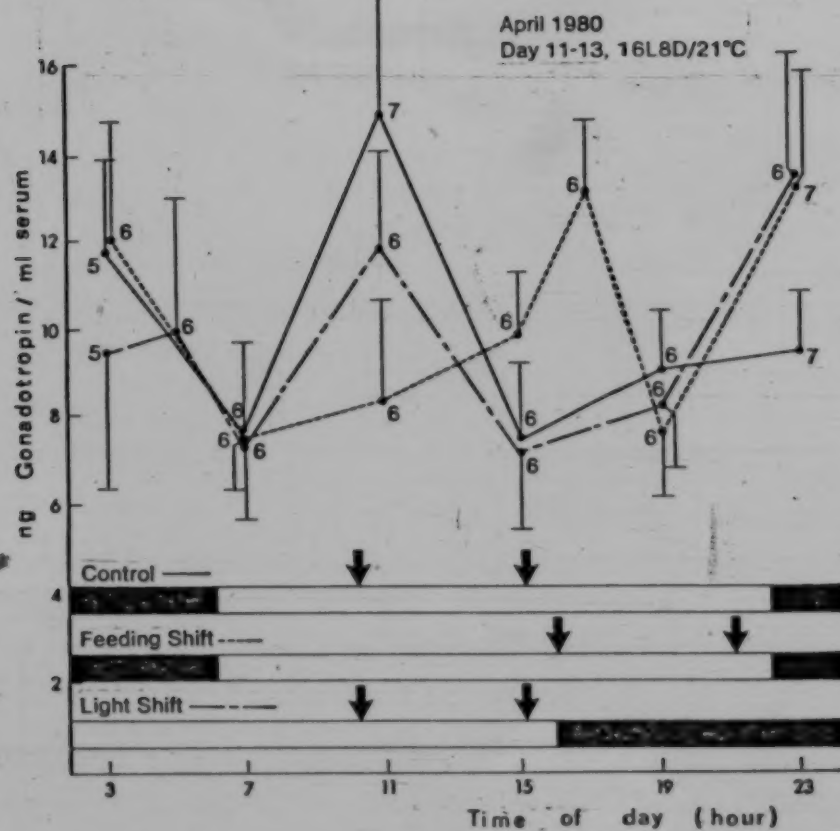
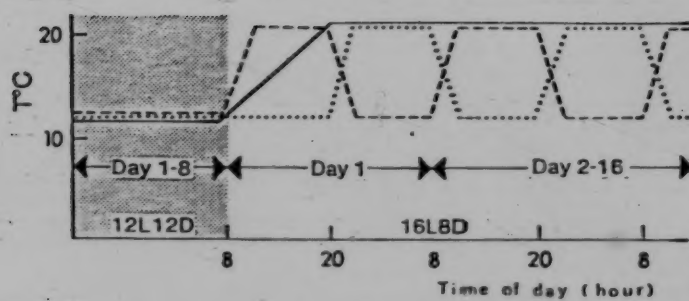


Figure 2.4. Serum GTH levels (mean  $\pm$  SE, n) on days 14 to 16 in fish held under various experimental temperature regimes in February.

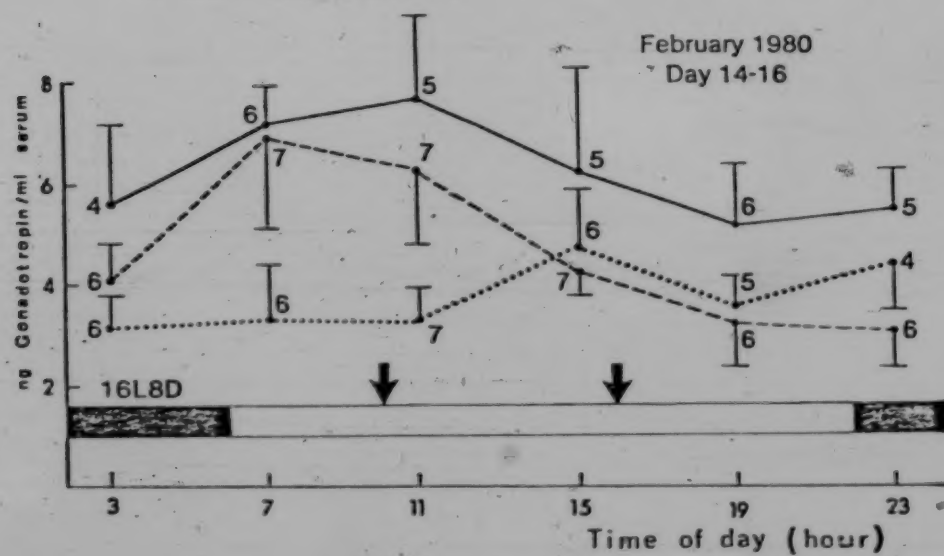
The temperature regimes and the results of the Duncan multiple range test ( $p < 0.05$ ) are shown on the upper half of the figure. The feeding times (indicated by arrows) and the photoperiod regimes are shown at the bottom of the figure.





|    |    |   |    |    |    |
|----|----|---|----|----|----|
| 19 | 23 | 3 | 15 | 7  | 11 |
| 23 | 19 | 3 | 15 | 11 | 7  |
| 3  | 11 | 7 | 19 | 15 | 23 |

Control ———  
 High Day - - - -  
 Low Day .....



## RESULTS

### I. Light and feeding shift experiment

Serum GTH levels in fish subjected for 11 to 13 days in April to a 16L8D/20  $\pm$  1<sup>0</sup> C regime in which both the onset of light and the two feeding times were shifted by 8 hours are shown in Figure 2.1. In the control group, a peak ( $p < 0.05$ ) in serum GTH was detected at 1100 h (5 hours after the onset of light or 1 hour after the first feeding). A similar peak ( $p < 0.05$ ) was found 8 hours later in the group subjected to the shift. The mean GSI values of the two groups were not significantly different.

### II. Light or feeding experiments

In an experiment done in November, a significant peak ( $p < 0.05$ ) in serum GTH levels was found at 1100h in the control group held under 16L8D/20  $\pm$  1<sup>0</sup> C for 5 to 7 days (Figure 2.2). (The pituitary GTH contents in this group were also determined and no significant fluctuations throughout the day were detected.) The serum GTH levels in the two groups in which either the feeding times or the onset of light were shifted by 6 hours were relatively low and uniform throughout the day. The mean GSI values of the three groups were not significantly different.

In an experiment done in April, a peak ( $p < 0.05$ ) in serum GTH levels was detected at 1100h in the control group subjected to the 16L8D/20  $\pm$  1<sup>0</sup> C regime for 11 to 13 days (Figure 2.3). Some fluctuations in serum GTH levels were evident in the two groups subjected to the feeding or the light shifts, but no statistically significant differences were found. The mean GSI values of the three groups were not significantly

different, and histological examination of the ovaries did not reveal any significant differences in cellular composition of the ovaries (Table 2.1).

The body weights of the fish at the start of the experiments in either November or April were not significantly different from the weights recorded at the end of the experiments.

### III. Temperature experiments

The experimental temperature regimes and the serum GTH levels in fish subjected to these regimes are shown in Figure 2.4. In the control group subjected to  $20 \pm 1^{\circ}\text{C}$  for 14 to 16 days in February, the serum GTH levels throughout the day were relatively high and no significant fluctuations were detected. In the group exposed to warm temperature only during the day ("high day" group), serum GTH levels found in the early portion of the light phase were higher ( $p < 0.05$ ) than the levels detected at the end of the light phase and early in the dark phase. Serum GTH levels in the group subjected to warm temperature only during night ("low day" group) were relatively low and uniform throughout the day.

The mean GSI values of the three experimental groups were not significantly different; however, some differences in the cellular composition of the ovaries were detected by histology (Table 2.2). A greater proportion ( $p < 0.005$ ) of ovaries from the control group were severely atretic compared to the "high day" group. A comparison of the control and the "low day" group revealed a trend ( $p < 0.1$ ) for a greater proportion of severely atretic ovaries in the control group. Also, ovaries from the control and "low day" groups tended ( $p < 0.2$ ) to have fewer

Table 2.1: Cellular composition of ovaries in fish in the light and/or feeding shift experiments in April, 1980.

| Experimental Group  | GSI<br>( $\bar{x} \pm SE$ ) | Number of cells/mm <sup>2</sup> of ovary ( $\bar{x} \pm SE$ ) |                    |                                              |                           | Number of severely atretic ovaries<br>(% of total number in a group) |
|---------------------|-----------------------------|---------------------------------------------------------------|--------------------|----------------------------------------------|---------------------------|----------------------------------------------------------------------|
|                     |                             | Perinucleolus Stage                                           | Yolk Vesicle Stage | 1 <sup>0</sup> and 2 <sup>0</sup> Yolk Stage | 3 <sup>0</sup> Yolk Stage |                                                                      |
| Control group       | 9.7±0.7<br>n=31             | 7.7±1.4<br>n=31                                               | 0.8±0.2            | 3.1±0.7                                      | 1.5±0.2                   | 42                                                                   |
| Feeding shift group | 10.1±0.8<br>n=37            | 9.0±1.2<br>n=37                                               | 0.9±0.1            | 3.5±0.4                                      | 1.7±0.2                   | 35                                                                   |
| Light shift group   | 10.4±0.6<br>n=35            | 10.2±2.0<br>n=34                                              | 0.7±0.1            | 2.4±0.3                                      | 1.8±0.2                   | 41                                                                   |



Table 2.2: Cellular composition of ovaries in fish in the temperature experiments in February, 1980.

| Experimental Group | GSI<br>( $\bar{x} \pm SE$ ) | Number of cells/mm <sup>2</sup> of ovary ( $\bar{x} \pm SE$ ) |                    |                                              |                           | Number of severely atretic ovaries<br>(% of total number in a group) |
|--------------------|-----------------------------|---------------------------------------------------------------|--------------------|----------------------------------------------|---------------------------|----------------------------------------------------------------------|
|                    |                             | Perinucleolus Stage                                           | Yolk Vesicle Stage | 1 <sup>0</sup> and 2 <sup>0</sup> Yolk Stage | 3 <sup>0</sup> Yolk Stage |                                                                      |
| Control            | 4.5±0.6<br>n=31             | 37.3±5.5                                                      | 2.4±0.3            | 5.9±0.6 <sup>a</sup>                         | 0.96±0.2 <sup>d</sup>     | 42 <sup>f</sup>                                                      |
| High Day           | 4.7±0.5<br>n=38             | 30.6±4.4                                                      | 2.1±0.3            | 7.2±0.6 <sup>b</sup>                         | 0.81±0.2                  | 11 <sup>g</sup>                                                      |
| Low day            | 4.6±0.5<br>n=35             | 36.0±5.8                                                      | 2.6±0.7            | 6.0±0.5 <sup>c</sup>                         | 0.5±0.2 <sup>e</sup>      | 23 <sup>h</sup>                                                      |

b > a, c (p < 0.2); d > e (p < 0.2); f > g (p < 0.005); f > h (p < 0.1)

oocytes in the 3<sup>0</sup> yolk stage than the control group.

#### DISCUSSION

Both the LD cycle and feeding times entrained the single mid-day peak in serum GTH levels in fish held under long photoperiod and warm temperature when the light and the feeding cues were given in a particular temporal synchrony. The temperature experiments suggested that the effects of warm temperature on GTH levels depend on the time of day of exposure. While warm temperature imposed during the photophase promoted a daily cycle in serum GTH levels and stimulated ovarian development, constant warmth or warm temperature given only during scotophase was correlated with uniform GTH levels throughout the day and atresia of oocytes.

A simultaneous shift in the onset of light and in the two feeding times imposed in April, 1979 shifted the pattern of the daily cycle in serum GTH levels in fish in the latter stages of ovarian recrudescence. The characteristic mid-day peak in serum GTH was detected 5 hours after the onset of light (or 1 hour after the first feeding) in the control group held under the 16L8D/20<sup>0</sup> C regime for 11 to 13 days. The peak was found about 8 hours later in the group subjected to the 8 hour shift in photoperiod and feeding. The LD cycle and/or feeding could therefore be considered as entraining cues used by the fish to regulate the pattern of the daily cycle in serum GTH levels.

The role of feeding in entrainment of GTH cycles has not been investigated previously, despite the evidence that there are other examples of daily cycles in hormone levels in teleosts being influenced by feeding

times (see Introduction). Stacey *et al.* (1979b) showed that the timing of spontaneous ovulations, and therefore also the preovulatory GTH surge in the goldfish, could be adjusted to a reversed photoperiod within 2 weeks. However, since fish were fed during the light phase under both the normal and reversed photoperiod, the contribution of feeding to the entrainment of the ovulation cycle to the new photoperiod was not determined.

The relative importance of the LD cycle and feeding in the entrainment of the daily cycle in serum GTH levels was investigated by imposing a 6 hour shift either in the onset of light or in the feeding times, in order to determine if photoperiod or feeding alone could entrain the GTH cycle. A single mid-day peak in serum GTH levels was detected 5 hours after the onset of light or 1 hour after the first feeding in fish in early recrudescence held under the 16L8D/20<sup>0</sup> C regime for 5 to 7 days in November, 1979. These results agree with the evidence presented in Chapters 1 and 4, since similar patterns in serum GTH levels were observed in fish subjected to the same regime in other experiments. However, a shift in either the onset of light or in the feeding times abolished the mid-day GTH peak. Clearly, the LD cycle alone does not entrain the GTH cycle, since fish were sampled 5 hours after the onset of light in both shifted groups and a peak in serum GTH levels was not found. One of the shifted groups was also sampled 1 hour after the first feeding, and the low GTH levels detected at that time suggest that feeding alone does not entrain the GTH cycle either. It was shown in the April, 1979 experiment that the GTH cycle was completely shifted by a simultaneous shift in the onset of light and feeding when the fish were

fed for the first time 4 hours after the lights went on. A simultaneous shift in these cues was also in fact imposed on the two shifted groups in November, 1979 and April, 1980 experiments. However, the fish were fed for the first time 10 hours after the onset of light in the shifted groups, and a daily cycle in serum GTH levels was not detected. Perhaps stress and/or decreased food consumption resulting from altered feeding schedules might be involved in the abolition of the GTH cycle in the shifted groups. However, since the weight gain of these two groups and the control groups was similar, it may be assumed that the fish in the shifted groups fed as much as the controls and, therefore, differential food consumption was probably not involved in the abolition of the GTH peak. Therefore, it seems that entrainment to the LD cycle and feeding is only possible when the cues are given in a particular temporal synchrony with each other.

The acclimation period in the November light or feeding shift experiments was 5 to 7 days, and it is possible that this length of time was insufficient for the entrainment to the shifted LD cycle and feeding to occur. Therefore, in April, 1980, fish were subjected to the same shifts in photoperiod and feeding as in November, but they were held under these regimes for 11 to 13 days. In the control (unshifted) group, a mid-day peak in serum GTH levels was found, as previously reported, in mature fish subjected to the 16L8D/20<sup>0</sup> C regime (Chapters 1 and 4; Hontela and Peter, 1978, 1980). Although a significant daily GTH cycle was not detected in the shifted groups, similar to the results in the experiment carried out in November with 5 to 7 days acclimation time, a great degree of fluctuation in serum GTH levels was observed in these groups.



However, since the fluctuations were not statistically significant and not consistently related to the onset of light or feeding, the data remain inconclusive. Therefore, although photoperiod and feeding are known to be important in the regulation of daily cycles in serum GTH levels, the relative importance of the cues cannot be evaluated by the present data. Histological examination showed no differences in the cellular composition of the ovaries of the control and the shifted groups in this experiment (April, 1980), although, based on previously reported data, some atresia might have been expected in the shifted groups if daily cycles in serum GTH levels were truly absent.

The influence of the time of the day of exposure to warm temperature on serum GTH levels was investigated in February, 1980. Exposure to a constant warm temperature and the 16L8D regime for 16 days resulted in uniform, relatively high serum GTH levels throughout the day, similar to the levels found in fish in early recrudescence held under 16L8D/20° C for 11 to 13, or 30 to 32 days in November (Chapter 1). Presumably, after exposure to 16L8D/20° C for 14 to 16 days, daily fluctuations in serum GTH levels were abolished as the nadir serum GTH levels increased to apogee levels. The data are compared to the November experiment rather than the March experiment because histological examination of ovaries suggested that these fish (in February) had not completed ovarian recrudescence, similar to the fish in November. The sinusoidal warm temperature regime lowered the mean serum GTH levels compared to the group held under a constant warm temperature regime, but the effects of the diurnal temperature fluctuations on GTH levels depended on the time of the day of exposure. Warm temperature imposed only during the photophase promoted a significant daily fluctuation in serum GTH levels.

the highest levels being found around the onset of light and first feeding. However, warm temperature during the scotophase resulted in uniform GTH levels. The relatively lower serum GTH levels found in fish subjected to only 12 hours of warm temperature each day compared to fish held under constant warmth are consistent with the stimulatory effect of warm temperature in serum GTH levels in many cyprinids, including the goldfish (for review, see Peter, 1981). However, the finding that warm temperature imposed during the photophase promoted a daily cycle in serum GTH levels, while warmth during the scotophase did not, suggests that a daily rhythm in responsiveness of the hypothalamo-pituitary axis to warm temperature exists in the goldfish. Some support for this hypothesis is provided by Spieler *et al.* (1977) who investigated the effects of 4 hour pulses of warm temperature imposed at six different times of the day on gonadal size in goldfish. Although no consistent trends were found in the changes of ovarian weights, testicular growth was stimulated more by warm temperature given during the last 4 hours of darkness than at any other time. Histological data provided by the present study indicated that ovaries of fish with fluctuating serum GTH levels were less atretic than ovaries of fish whose GTH levels were constant during the day. This is consistent with data presented in Chapter 1 and the hypothesis that fluctuating serum GTH levels are important for ovarian development in the goldfish. Interestingly, the steady and lower GTH levels found in the group exposed to warmth during the night resulted in a smaller percentage of atretic ovaries than the steady GTH levels of the group held under constant warmth. Whether this difference is related to the different mean serum GTH levels throughout the day in the two

groups or to differences in temporal changes in the patterns of the daily cycles in serum GTH levels throughout the acclimation period is not known.

Photoperiod, feeding times and temperature are environmental cues used in the regulation of patterns of the daily cycles in serum GTH levels in the goldfish. The present study demonstrated, for the first time in a teleost fish, that the GTH cycle can be entrained by the LD cycle and feeding times, when these cues are given in a particular temporal synchrony. Since the effects of warm temperature in inducing a daily cycle in serum GTH depended on the time of day of exposure, the target organs within the reproductive axis of the goldfish seem also to have a daily rhythm in responsiveness to temperature. Information concerning daily fluctuations in responsiveness of the reproductive system of teleosts to environmental cues is limited. The effects of feeding have not been investigated at all, and the above mentioned study by Spieler *et al.* (1977) is the only published report concerning daily variations in the effects of warm temperature on gonadal growth. The effects of photoperiod have been studied more thoroughly. It has been shown that 1 hour of light given to medaka (Chan, 1976) or catfish (Sundararaj and Vasal, 1976) held under non-stimulatory photoperiods stimulated the reproductive system, as indicated by accelerated gonadal growth, if the light pulse was given at specific times of the day. Similar experiments have been carried out with hamsters (Hoffman, 1979; Rudeen and Reiter, 1980), ferrets (Boissin-Agasse and Ortavant, 1978), rams (Garnier *et al.*, 1977) and birds (Hamner, 1963; Follett *et al.*, 1974), although indices within the reproductive system other than gonadal

size were measured in some of these studies. The data are thought to indicate the existence of daily rhythms in photosensitivity; only light given during the photosensitive phase of the day photostimulates the system under study. A circadian clock seems to be involved in the photoperiodic time measurements in birds. Although such a circadian clock apparently exists in some teleosts (for review, see Kavaliers, 1979), data provided by this study do not suggest that such a clock exists for the daily cycle in serum GTH levels in goldfish, since the cycle disappears after at most 16 days exposure to a rhythmic environment.

At the present time, it is only possible to speculate about the physiological significance of a daily cycle in responsiveness of the hypothalamo-pituitary axis of the goldfish to feeding and temperature. Since it seems that only cues perceived at certain times of the day promote fluctuations in serum GTH levels, and that such fluctuations stimulate ovarian development (Chapters 1 and 4; Hontela and Peter, 1978, 1980), daily cycles in responsiveness to feeding and temperature may allow the fish to use these cues in a physiologically advantageous manner. It is conceivable that by feeding and by being exposed to warm temperature at specific times of the day, fluctuations in serum GTH levels are induced and ovarian development might be promoted. Daily cycles in responsiveness of the gonads to GTH have been demonstrated in the shiner (de Vlaming and Vodcnik, 1977) and the goldfish (Peter *et al.*, 1982). Other hormones (e.g. thyroid hormones or corticosteroids) or metabolites (e.g. glucose, glycogen, triglycerides) may act synergistically with GTH or be required for ovarian growth, and changes in blood



levels of these factors may also be influenced by the environmental cues (see Introduction). Daily fluctuations in blood levels of amino acids (Carillo *et al.*, 1980) and catecholamines (Sauerbier and Meyer, 1977) have been reported in goldfish; such factors and others could influence the responsiveness of the gonads to a pulse in blood levels of GTH.

Information concerning feeding and thermoregulatory behaviour of teleosts subjected to unrestricted environmental schedules or the natural regime is scarce. Field observations of feeding activity, and data on stomach contents of fish caught at certain times of day suggest that some species of fish feed at specific times of the day (for review, see Schwassmann, 1971). Eriksson (1975) reported that sea-run brown trout, kept at low temperature in the spring self-selected two feeding times during the day, at dawn and dusk. Eriksson and Van Veen (1980) also showed that feeding behaviour in the brown bullhead *Ictalurus nebulosus* is circadian but synchronized to the LD cycle. It is not known at present whether the feeding times shown to entrain the GTH cycle in the goldfish in the present study would be at or near self-selected feeding times. Several teleost fishes (Reynolds and Casterlin, 1978; Reynolds *et al.*, 1978a, 1978b), including the goldfish (Reynolds *et al.*, 1978b) have been shown to behaviourally thermoregulate in free choice laboratory experiments; goldfish chose the warmest temperatures near the onset of light. Although under certain circumstances temperature fluctuates throughout the day and spatial differences in temperature exist in shallow ponds where goldfish live, it has not been shown directly that goldfish behaviourally thermoregulate under these conditions.

Interestingly, the center for behavioural thermoregulation in some teleosts is situated in the preoptic region (Nelson and Prosser, 1979), an area also involved with the neuroendocrine control of reproduction (Peter, 1982). Data provided by experiments described in this chapter and those reported in the literature allow formulation of a working hypothesis: the ovarian development in the goldfish kept in a natural environment might be stimulated at certain times of the year by environmental cues, either self-selected or perceived in such a time sequence as to promote a daily cycle in serum GTH levels.

CHAPTER 3: EFFECTS OF PINEALECTOMY, BLINDING, AND SEXUAL CONDITION  
ON SERUM GONADOTROPIN LEVELS IN THE GOLDFISH.

(The text of this chapter is in the Appendix, page 161 as a reprint  
from General and Comparative Endocrinology 40: 168-179, 1980.)

## CHAPTER 4: EFFECTS OF MELATONIN ON GONADOTROPIN HORMONE LEVELS

### INTRODUCTION

The role of the pineal organ in regulation of gonadal development has been investigated in some species of spring-spawning teleost fishes. In such fish, the influence exerted by the pineal on the reproductive axis seems to depend on photoperiod, temperature and gonadal condition. Pinealectomy had no apparent effects on gonadal activity in sexually regressed fish (Fenwick, 1970a; Vodcnik *et al.*, 1978; Hontela and Peter, 1980). On the other hand, pinealectomy accelerated and inhibited gonadal development under short and long photoperiods, respectively, in both maturing and mature shiners, *Notemigonus crysoleucas* (de Vlaming, 1975), goldfish *Carassius auratus* (de Vlaming and Vodcnik, 1978; Vodcnik *et al.*, 1978; Hontela and Peter, 1980) and medaka, *Oryzias latipes* (Urasaki, 1973, 1976) subjected to warm temperature (see Chapter 3). The effects of pinealectomy on gonadal development have not been investigated in teleosts that accelerate their gonadal activity in response to a short or decreasing photoperiod. The effects of pinealectomy on GTH levels in the goldfish also depend on photoperiod, temperature and gonadal condition (Vodcnik *et al.*, 1978; Hontela and Peter, 1980). Hontela and Peter (1980; Chapter 3) demonstrated that in female goldfish having ovaries containing some oocytes that had completed vitellogenesis the pineal promoted daily fluctuations in serum GTH levels under long photoperiod and warm temperature, and suppressed them under short photoperiod and warm temperature in the spring. It is hypothe-



sized that a daily cycle in blood GTH levels is important in stimulation of ovarian development in the goldfish (Hontela and Peter, 1978; Chapters 1 and 2) and that the pineal influences gonadal maturation under various photoperiod and temperature regimes through, at least in part, alterations of the daily cycles in blood GTH levels.

The understanding of the mechanisms through which the pineal organ alters GTH levels is still limited, but the evidence that the pineal has important influences on, and is influenced by the hypothalamo-hypophysial-gonadal axis is accumulating rapidly. Melatonin (MT), a pineal hormone, can inhibit the pituitary response to LHRH in rat (Martin *et al.*, 1977, 1980; Bacon *et al.*, 1981); however, it has also been reported to stimulate the release of gonadotropin-releasing hormone from the isolated medial basal hypothalamus of the rat (Kao and Weisz, 1977). Recently, sites of antagonistic action of MT were identified in specific areas of the brain in the white-footed mouse, *Peromyscus leucopus* (Glass and Lynch, 1981). MT decreases blood levels of FSH and/or LH in mammals under most experimental regimes (for review, see Reiter *et al.*, 1978; Turek and Campbell, 1979; Reiter, 1980a). However, single daily MT injections had inhibitory influences on the gonadal functions in some mammalian species only when given in the afternoon, not in the morning (Tamarkin *et al.*, 1976; Reiter *et al.*, 1976; Margolis and Lynch, 1981). Furthermore, the effect of the afternoon MT injections was negated by MT administered either in subcutaneously implanted continuous-release capsules (Trakulrungrasi *et al.*, 1979; Reiter *et al.*, 1977) or by MT injected in the morning (Chen *et al.*, 1980). This apparently contradictory evidence has been recently reviewed by Reiter (1980b) who

theorized that the daily variations in sensitivity of the reproductive system of some mammals to MT may be related to fluctuations in number and/or activity of MT receptors within the reproductive axis, and down regulation of these receptors by MT.

MT has been identified in the pineal organ in goldfish (Fenwick, 1970b), and the pineal in rainbow trout has been shown to incorporate precursors for MT synthesis (Hafeez and Zerihun, 1976). Recently, the retina of the rainbow trout has been shown to synthesize MT *in vitro* (Gern and Ralph, 1979); the pineal was not investigated in this regard. Diurnal fluctuations in MT levels have been detected in the blood and the retina of rainbow trout; blood MT levels were highest during the scotophase portion of the photoperiod (Gern *et al.*, 1978a; Owens *et al.*, 1978), while MT in the retina was present in highest concentrations during the photophase (Gern *et al.*, 1978b). MT levels in the pineal were not determined.

MT has been implicated in several physiological processes in teleost fishes. A melanin-aggregating (blanching) effect of MT on the skin has been described in several species (for example Reed *et al.*, 1969; Ruffin *et al.*, 1969; Hafeez, 1970). Swimming movements in goldfish (Satake, 1979) and rainbow trout (Hafeez, 1970) were altered by MT injections. In addition, effects of MT on linear growth, weight gain (de Vlaming, 1980) and carbohydrate and/or lipid metabolism (de Vlaming *et al.*, 1974b; Delahunty *et al.*, 1978b) were also reported. Interestingly, the effects of MT on growth and metabolism seemed to depend on the time of year and the photoperiod regime to which the fish were subjected; both stimulatory and inhibitory influences were detected.

MT also influences the reproductive axis in teleost fishes; treatment with MT had an inhibitory effect on gonadal development in the medaka (Urasaki, 1972, 1977), the catfish, *Heteropneustes fossilis* (Sundararaj and Keshavanath, 1976), and *Mustus tengara* (Saxena and Anand, 1977), the killifish, *Fundulus similis* (de Vlaming et al., 1974a), goldfish (Fenwick, 1970b) and three-spined stickleback, *Gasterosteus aculeatus* (Borg and Ekström, 1981). In this last study, MT has been found to stimulate gonadal activity in sticklebacks subjected to specific conditions of photoperiod and temperature in winter and summer. However, the effects of MT on GTH levels in a teleost fish have not been reported previously. In the present study, the effects of MT on serum GTH levels and gonadal development in goldfish were determined. Also, the effects of MT injections given at various times of the day on serum GTH levels were investigated.

## MATERIALS AND METHODS

See Materials and Methods of Chapter 1 for descriptions of experimental animals (section I), initial acclimation regime (section II), blood sampling technique (section III), radioimmunoassay for GTH (section IV), histology (section V) and statistical analysis (section VI).

### I. Experimental environmental regimes

Following the initial acclimation regime, fish were subjected to 12L12D/12  $\pm$  1° C for 8 days, then to 16L8D/20  $\pm$  1° C for various periods of time depending on the experiments. Light was turned on at 0600h and fish were fed at 1000h and 1600h in all the experiments.

## II. Operations

Pinealectomized fish were used in some experiments. The techniques for pinealectomy and sham surgery are described in detail by Hontela and Peter (1980; Chapter 3).

## III. Injection technique

MT (Sigma) was weighed, dissolved in 0.5 ml of 95% ethyl alcohol, and mixed with teleost physiological saline 30 minutes before the injection. The saline with 95% alcohol was used as control solution; both solutions were kept on cracked ice and in the dark until use. Fish were anaesthetized with tricaine methane sulfonate (1:1000) until swimming ceased but opercular movements were still detectable, and injected intraperitoneally. The site of injection was then covered with a thin layer of Orabase gel (Squibb Canada Inc., Montreal, Canada).

## IV. Injection and sampling technique

### May, 1979 experiment

Fish were pinealectomized or sham operated on the first day of the 12L12D/20  $\pm$  1 $^{\circ}$  C regime. Pinealectomized fish were injected with MT (1  $\mu$ g/g body weight/5  $\mu$ l saline) or saline, and the sham operated fish were injected with saline between 2000h and 2130h on days 6, 7 and 8 of the 16L8D/20  $\pm$  1 $^{\circ}$  C regime. Fish were blood sampled at 1100h on day 9. Ovary and body weights were determined for calculation of the GSI's. Ovaries were examined histologically.



#### April, 1980 experiment

Intact fish were injected with MT ( $12 \mu\text{g/g}$  body weight/ $10 \mu\text{l}$  saline) or saline once a day between 1600h and 1800h. They received four injections under the  $12\text{L}12\text{D}/12 \pm 1^\circ \text{C}$  regime and fourteen injections under the  $16\text{L}8\text{D}/20 \pm 1^\circ \text{C}$  regime. Blood was sampled at 1100h and 1900h on day 14 of the  $16\text{L}8\text{D}/20 \pm 1^\circ \text{C}$  regime. After sampling at 1900h, body and ovary weights were determined for calculation of GSI's.

#### November, 1980 experiment

Intact fish were injected with MT ( $10 \mu\text{g/g}$  body weight/ $10 \mu\text{l}$  saline) or saline at 0700h on days 5, 6 and 7 or at 1500h on days 4, 5 and 6 of the  $16\text{L}8\text{D}/20 \pm 1^\circ \text{C}$  regime. All fish were sampled at 1100h and 1900h on day 7. The fish were left under the  $16\text{L}8\text{D}/20 \pm 1^\circ \text{C}$  regime. They were injected with the same solutions as on days 4 to 7 at 0930h on day 11 and sampled at 1100h and 1900h on the same day. Ovaries were examined histologically.

#### February, 1981 experiment

Intact fish were injected with MT ( $10 \mu\text{g/g}$  body weight/ $10 \mu\text{l}$  saline) or saline at 0700h on days 5, 6 and 7 of the  $16\text{L}8\text{D}/20 \pm 1^\circ \text{C}$  regime. They were sampled at 1100h on day 7. Body and ovary weights were determined for calculation of GSI's.

### RESULTS

#### I. May, 1979

Serum GTH levels in pinealectomized and sham operated fish injected with MT ( $1 \mu\text{g/g}$  body weight/ $5 \mu\text{l}$  saline) or saline 1 hour before dark on days 6, 7 and 8 of the  $16\text{L}8\text{D}/20 \pm 1^\circ \text{C}$  regime are shown in Figure

4.1. GTH levels detected 5 hours after the onset of light on day 9 were higher ( $p < 0.05$ ) in sham operated fish injected with saline than in pinealectomized fish injected either with saline or MT. No significant differences between the mean GSI values of the three groups were found; however, histological examination of the ovaries (Table 4.1) showed that the ovaries of the sham operated group had more oocytes in the 3<sup>0</sup> yolk stage than the ovaries of the pinealectomized saline ( $p < 0.01$ ) or MT injected ( $p < 0.1$ ) groups. Furthermore, a significantly smaller proportion of fish with severely atretic ovaries ( $p < 0.05$ ) was found in the sham operated group than in the two pinealectomized groups.

## II. April, 1980

Serum GTH levels in intact fish injected once a day for 18 days with MT (12  $\mu\text{g/g}$  body weight/10  $\mu\text{l}$  saline) or saline 5 hours before dark are shown in Figure 4.2. GTH levels were determined at 5 and 13 hours after the onset of light on day 14 of the 16L8D/20  $\pm$  1<sup>0</sup> C regime in both groups; no significant differences between any of the means were found. The mean GSI values of the saline and MT treated fish were not significantly different (data not shown).

## III. November, 1980

Serum GTH levels in intact fish injected with MT (10  $\mu\text{g/g}$  body weight/10  $\mu\text{l}$  saline) or saline either 1 hour after the onset of light on days 5, 6 and 7 (AM injection), or 9 hours after the onset of light on days 4, 5 and 6 (PM injection) of the 16L8D/20  $\pm$  1<sup>0</sup> C regime are shown in Figure 4.3a. The saline treated fish injected in the morning (AM injection) had higher serum GTH levels ( $p < 0.05$ ) at 1100h than

Figure 4.1. Serum GTH levels (mean  $\pm$  SE, n) at 1100h on day 9 of the 16L8D/20  $\pm$  1  $^{\circ}$ C (light on at 0600h) regime in pinealectomized (PNX) and sham operated fish injected with melatonin or saline at 2100h on days 6, 7 and 8.

Significant differences by Student's t-test ( $p < 0.05$ ) are indicated beside the histograms (\*).

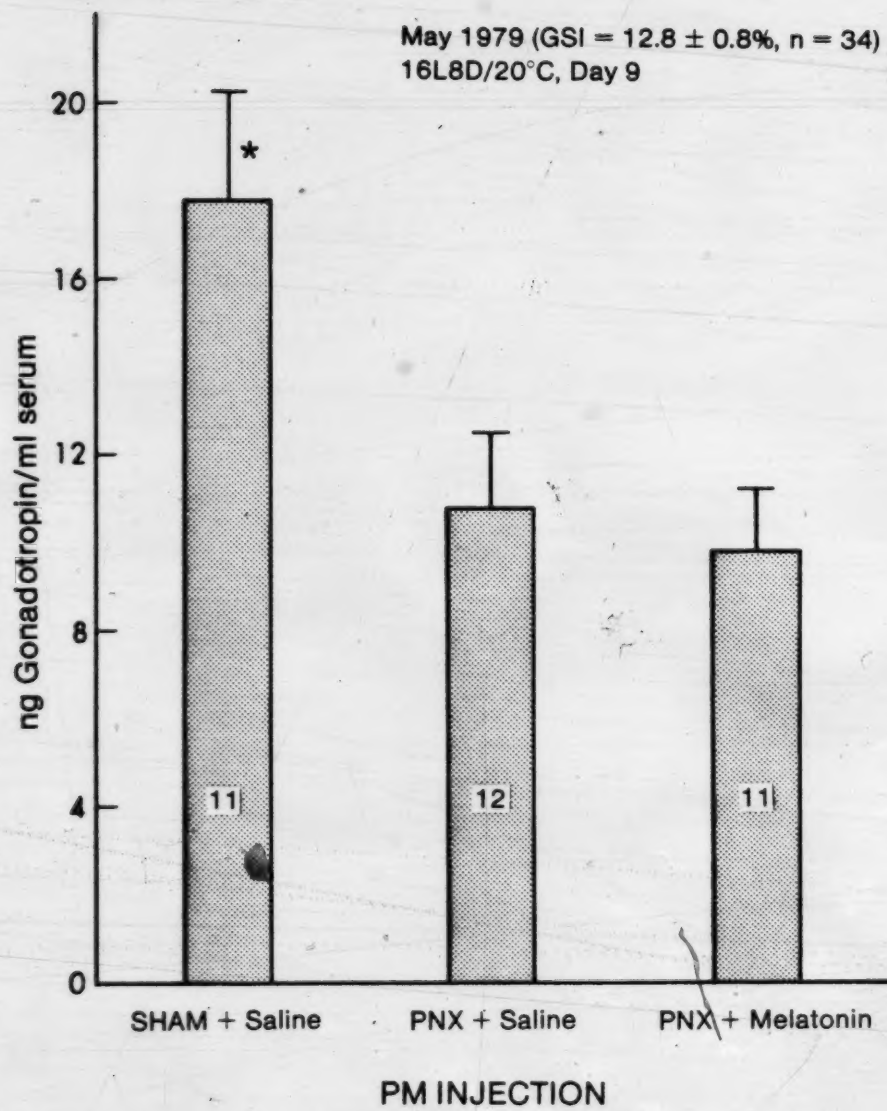




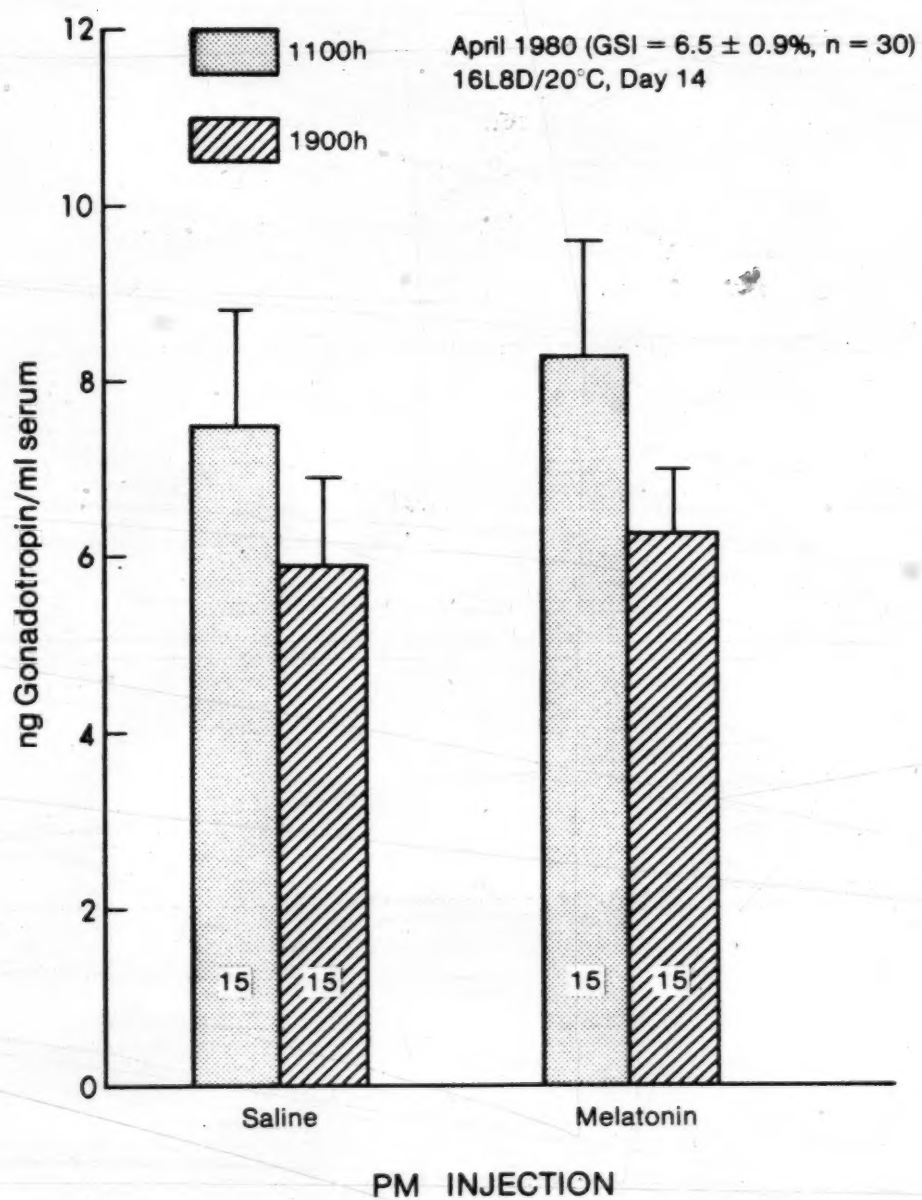
Table 4.1: Cellular composition of ovaries in fish from the May, 1979 pinealectomy and melatonin injection experiment.

| Experimental Group          | GSI<br>( $\bar{x} \pm SE$ ) | Number of cells/mm <sup>2</sup> of ovary ( $\bar{x} \pm SE$ ) |                    |                                              |                           | Number of severely atretic ovaries<br>(% of total number in a group) |
|-----------------------------|-----------------------------|---------------------------------------------------------------|--------------------|----------------------------------------------|---------------------------|----------------------------------------------------------------------|
|                             |                             | Perinucleolus Stage                                           | Yolk Vesicle Stage | 1 <sup>0</sup> and 2 <sup>0</sup> Yolk Stage | 3 <sup>0</sup> Yolk Stage |                                                                      |
| Sham operated + Saline      | 11.9±0.8<br>n=14            | 4.8±1.1<br>n=10                                               | 0.5±0.1            | 2.1±0.5                                      | 3.2±0.12 <sup>a</sup>     | 10*                                                                  |
| Pinealectomized + Saline    | 12.6±0.8<br>n=12            | 5.6±0.5<br>n=12                                               | 0.9±0.2            | 2.9±0.3                                      | 2.7±0.12 <sup>b</sup>     | 67                                                                   |
| Pinealectomized + Melatonin | 14.5±1.1<br>n=11            | 4.8±0.8<br>n=11                                               | 0.5±0.1            | 2.6±0.4                                      | 2.7±0.18 <sup>c</sup>     | 55                                                                   |

a > b (p < 0.01); a > c (p < 0.1); \*smallest proportion of severely atretic ovaries found in this group (p < 0.05)

Figure 4.2. Serum GTH levels (mean  $\pm$  SE, n) at 1100h on day 14 of the 16L8D/20  $\pm$  1  $^{\circ}$ C (light on at 0600h) regime in intact fish injected with melatonin or saline once a day at 1700h (18 injections in total).

No significant differences by Student's t-test ( $p < 0.05$ ) were found.

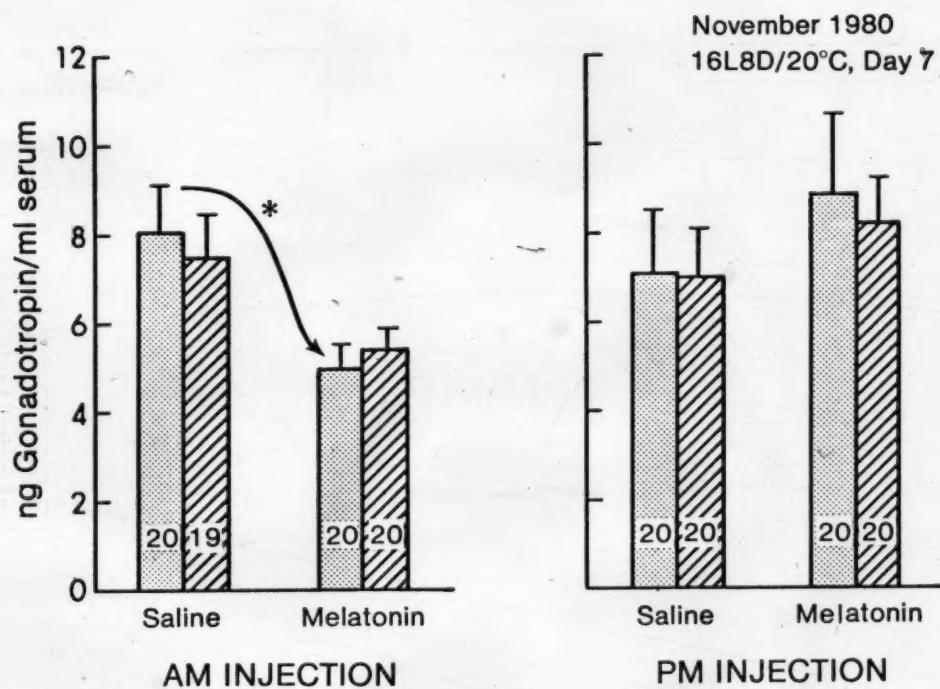


- Figure 4.3. a) Serum GTH levels (mean  $\pm$  SE, n) at 1100h and 1900h on day 7 of the 16L8D/20  $\pm$  1 $^{\circ}$  C (light on at 0600h) regime in intact fish injected with melatonin or saline either at 0700h on days 5, 6 and 7, or at 1500h on days 4, 5 and 6.
- b) Serum GTH levels (mean  $\pm$  SE, n) at 1100h on day 11 of the 16L8D/20  $\pm$  1 $^{\circ}$  C (light on at 0600h) regime in intact fish injected with melatonin or saline either at 0700h on days 5, 6 and 7 or at 1500h on days 4, 5 and 6, and at 0930h on day 11.

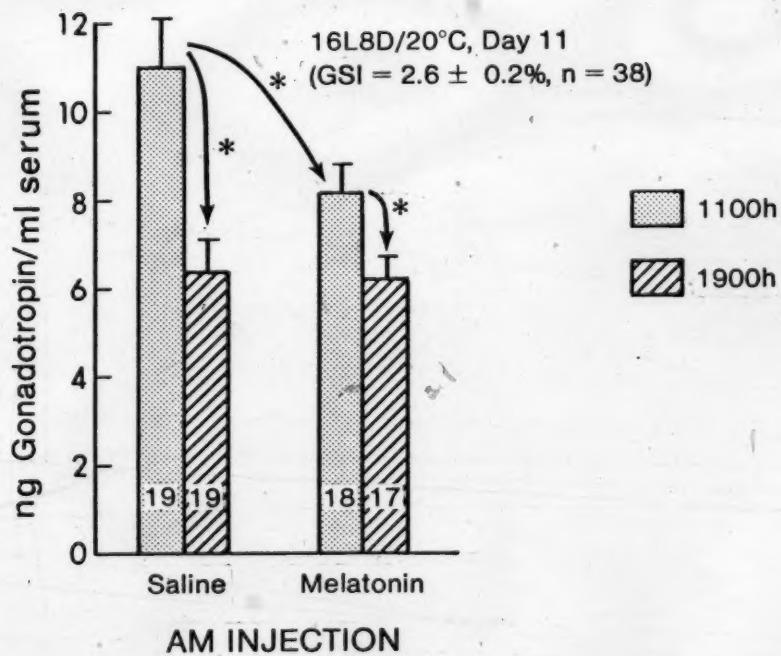
Significant differences by Student's t-test ( $p < 0.05$ ) are indicated beside the histogram (\*).



A



# B



the MT treated fish injected in the morning. Serum GTH levels at 1100h in the saline treated fish and the MT treated fish injected in the afternoon ( PM injection ) were not significantly different. Serum GTH levels at 1100h were not significantly different from GTH levels at 1900h in the fish treated with saline or MT in the morning or in the afternoon.

Serum GTH levels in intact fish which, subsequently to injection with either saline or MT throughout days 4 to 7, were again injected with MT (10  $\mu$ g/g body weight/10  $\mu$ l saline) or saline 1½ hour after the onset of light on day 11 of the 16L8D/20  $\pm$  1° C regime are shown in Figure 4.3b. The saline injected fish had higher ( $p < 0.05$ ) GTH levels than the MT injected fish at 1100h on day 11. Also, the GTH levels at 1100h were higher ( $p < 0.05$ ) than the GTH levels at 1900h in both experimental groups. The mean GSI values and the cellular composition of the ovaries (Table 4.2) of the saline and MT treated fish were not significantly different.

#### IV. February, 1981

Serum GTH levels in intact fish injected with MT (10  $\mu$ g/g body weight/10  $\mu$ l saline) or saline 1 hour after the onset of light on days 5, 6 and 7 of the 16L8D/20  $\pm$  1° C regime are shown in Figure 4.4. The GTH levels 5 hours after the onset of light on day 7 in the saline and MT treated groups were not significantly different. There were no significant differences in mean GSI values between the two groups (data not shown).

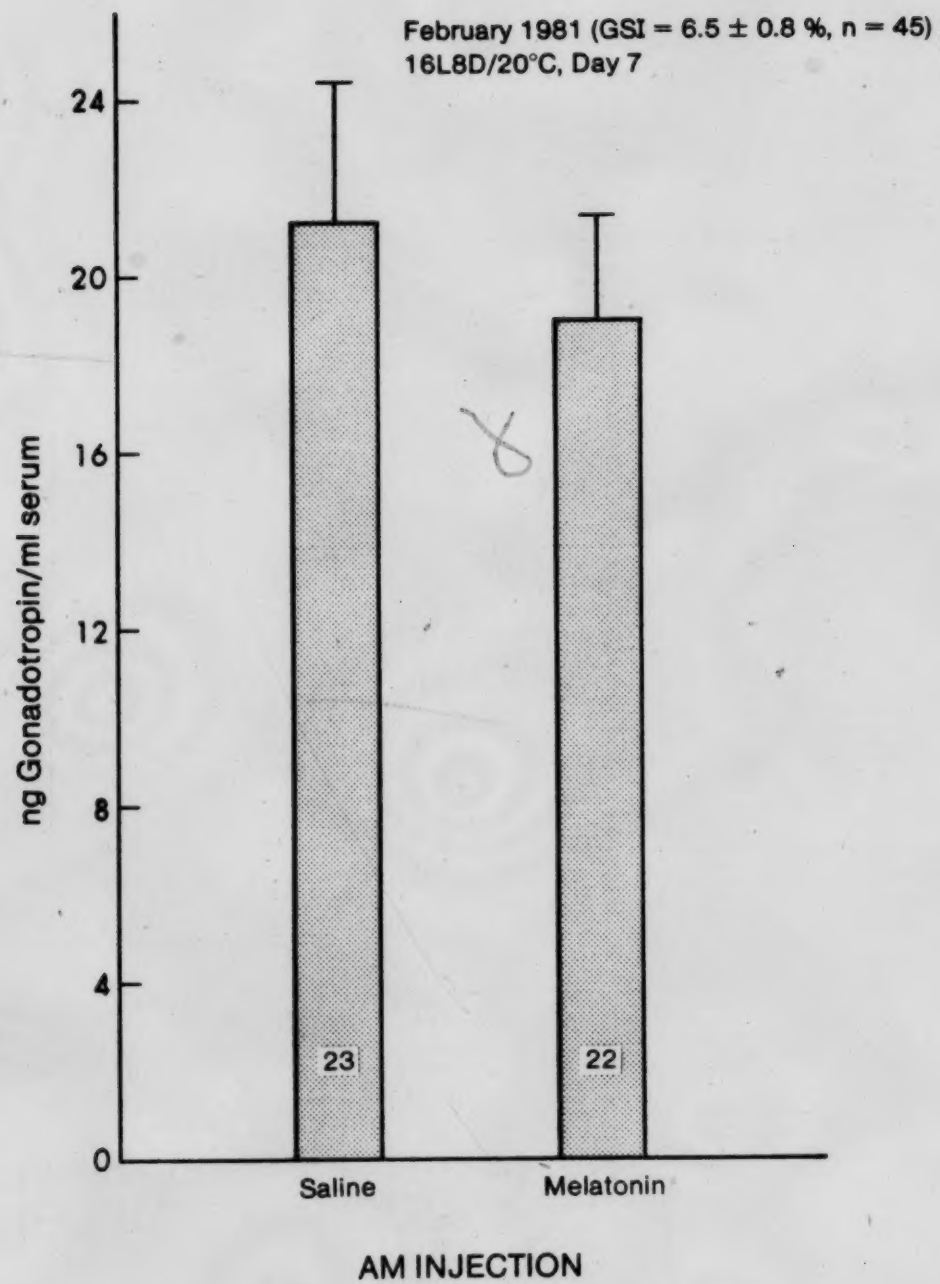
Table 4.2: Cellular composition of ovaries in fish from the November, 1980 melatonin injection experiment.

| Experimental Group | GSI<br>( $\bar{x} \pm SE$ ) | Number of cells/mm <sup>2</sup> of ovary ( $\bar{x} \pm SE$ ) |                    |                                              | Number of severely atretic ovaries<br>(% of total number in a group) |
|--------------------|-----------------------------|---------------------------------------------------------------|--------------------|----------------------------------------------|----------------------------------------------------------------------|
|                    |                             | Perinucleolus Stage                                           | Yolk Vesicle Stage | 1 <sup>0</sup> and 2 <sup>0</sup> Yolk Stage |                                                                      |
| Saline injected    | 2.5±0.2<br>n=19             | 57.9±6.8<br>n=18                                              | 6.4±0.8            | 13.6±0.9                                     | 0                                                                    |
|                    |                             |                                                               |                    |                                              | 11                                                                   |
| Melatonin injected | 2.8±0.3<br>n=18             | 52.1±6.2<br>n=14                                              | 6.2±0.6            | 14.2±0.9                                     | 0                                                                    |
|                    |                             |                                                               |                    |                                              | 7                                                                    |

Figure 4.4. Serum GTH levels (mean  $\pm$  SE, n) at 1100h on day 7 of the 16L8D/20  $\pm$  1  $^{\circ}$ C (light on at 0600h) regime in intact fish injected with melatonin or saline at 0700h on days 5, 6 and 7.

No significant differences by Student's t-test ( $p < 0.05$ ) were found.





## DISCUSSION

The present study confirms results reported in Chapter 3 (Hontela and Peter, 1980) which demonstrated that pinealectomy lowers the mid-day serum GTH levels and inhibits ovarian development in female goldfish in the latter stages of ovarian recrudescence ("spring" fish) subjected to long photoperiod and warm temperature. More importantly, this study indicates that, under this environmental regime, MT injected in the morning decreased serum GTH levels in intact fish in early stages of ovarian recrudescence ("winter" fish) but had no effect in "spring" fish. MT administered in the afternoon had no effect in intact "winter" fish, or in intact or pinealectomized "spring" fish.

The experiment carried out in May, 1979 showed that the serum GTH levels detected 5 hours after the onset of light (1100h) were lower in pinealectomized fish injected with saline or MT than in sham operated, saline injected fish subjected to 16L8D/20<sup>0</sup> C for 9 days. The histological examination of the ovaries revealed that the pinealectomized fish had fewer mature yolky oocytes and more atresia than the sham operated fish, although no effect on GSI was evident; in previous work (Hontela and Peter, 1980; Chapter 3) a significant decrease in GSI was found following pinealectomy of female goldfish in latter stages of ovarian recrudescence held under similar environmental conditions. Histological examination was not done to assess the ovarian condition by Hontela and Peter (1980), but presumably the decrease in GSI found in this work was due to oocyte atresia. The serum GTH levels were not determined at 1900h in the present study, and therefore it is not known

with certainty whether the daily fluctuations in serum GTH levels were abolished by pinealectomy. Nevertheless, these data indicate that the onset of oocyte atresia in female goldfish in the latter stages of ovarian recrudescence is accelerated when the mid-day serum GTH levels are decreased by pinealectomy. The results in this chapter confirm that the pineal has a stimulatory or progonadal influence under long photoperiod and warm temperature in mature female goldfish.

Inhibitory effects of pinealectomy on gonadal development have been reported in teleost fishes held under long photoperiod and warm temperature (see Introduction of Chapters 3 and 4); and also in birds (Saylor and Wolfson, 1967) and mammals (Herbert, 1971; Hoffman and Küderling, 1975; Bittman and Zucker, 1981) under long photoperiod conditions. However, the mechanisms by which the pineal mediates its stimulatory effects on the reproductive axis under long photoperiod are not understood. Although inhibitory influences of MT on GSI have been found in most studies on teleost fish (see Introduction of Chapter 4), MT has also been reported to stimulate gonadal function in the three-spined stickleback (Borg and Ekström, 1981), and in mammals (Reiter *et al.*, 1975; Turek *et al.*, 1975) under certain experimental conditions. Since the effects of MT on GTH levels have not been investigated in a teleost fish, a replacement therapy experiment was carried out in May, 1979 in order to determine whether MT is a mediator of the progonadal influence of the pineal under the 16L8D/20<sup>0</sup> C regime. A nocturnal rise of plasma MT levels has been detected in intact rainbow trout (Gern *et al.*, 1978a; Owens *et al.*, 1978), and, although MT levels have not been determined in the goldfish, daily fluctuations of some morpholog-

ical parameters of the pineal indicate that in goldfish subjected to the 16L8D/20<sup>0</sup> C regime, the pineal metabolic activity, a possible indicator of secretory activity, is highest in mid-scotophase (McNulty, 1981). To simulate the nocturnal rise of plasma MT levels, three single daily MT injections were given at one hour before the onset of dark to pinealectomized fish held under 16L8D/20<sup>0</sup> C. MT, however, had no effect on GTH levels in pinealectomized fish under these experimental conditions.

A single daily injection of MT alters the reproductive axis in mammals usually only if an intact pineal is present (Reiter *et al.*, 1976), although data indicating an effect of MT in pinealectomized hamsters has been also reported (Hoffman and Kuderling, 1977). In all the investigations in which an effect of MT on gonadal development in fish was demonstrated (see Introduction of Chapter 4) the pineal was intact. Thus, it may be possible that MT has no effects on GTH levels in pinealectomized teleosts. Although the injected dose of MT (1 µg/g body weight) used in the above experiment could have been too low, Fenwick (1970b) reported that daily injections of a similar dose of MT caused a significant decrease in GSI in goldfish. It has been reported that the mammalian reproductive system could be inhibited by MT injected in the afternoon, but not by injections at other times of the day (Reiter *et al.*, 1976; Tamarikin *et al.*, 1976; Margolis and Lynch, 1981). Studies which demonstrated an inhibitory effect of MT on the gonadal development in teleosts do not indicate an optimal time for MT injections; some investigators did not report the time of injection (Fenwick, 1970b; Urasaki, 1977), some injected the fish in the morning (Saxena



and Anand, 1977; de Vlaming *et al.*, 1974a) and others in the afternoon (Sundararaj and Keshavanath, 1976; de Vlaming *et al.*, 1974a). In all of these investigations, the fish were injected daily over relatively long periods of time (50 injections by Fenwick (1970b); 10 to 15 injections by de Vlaming *et al.* (1974a); 20 injections by Sundararaj and Keshavanath (1976); 60 injections by Saxena and Anand (1977); 23 injections by Urasaki (1977)).

To determine the effects of daily injections of MT for an extended period, intact goldfish were injected daily in the afternoon with MT for 18 days in April, 1980. However there were no significant effects on GTH levels at 1100h or 1900h, compared to injections of saline, and there was no effect on GSI. In this experiment, the difference between serum GTH levels at 1100h and 1900h usually detected in intact fish in the latter stages of ovarian recrudescence subjected to the 16L8D/20<sup>0</sup> C regime for about two weeks in the spring (Chapters 1 and 2) was not found in the saline injected animals. Perhaps the repeated handling of the fish during the injections disrupted the daily cycle in serum GTH levels. Also, although data from the mammalian literature were convincing in promoting the idea that only afternoon injections of MT are effective in altering the reproductive axis, it seemed evident that even if a daily rhythm in responsiveness of the hypothalamo-hypophysial-gonadal axis to MT exists in the goldfish, the period of greatest responsiveness was not the afternoon.

To further investigate the possibility that there is a daily variation in responsiveness to MT, the effects of morning and afternoon MT

injections on GTH levels were compared in an experiment carried out in November. Fish in early stages of ovarian recrudescence were subjected to the 16L8D/20<sup>0</sup> C regime for 7 days, since intact fish held under such a regime have a peak in serum GTH levels at 1100h, while the GTH levels are low and uniform throughout the rest of the day (Chapter 1). Three morning injections of MT lowered the serum GTH levels at 1100h compared to saline injected fish sampled at 1100h. The serum GTH levels at 1900h were also lower in the MT injected group compared to the saline treated fish, but the difference was not statistically significant. MT administered in the afternoon had no effect on GTH levels at either 1100h or 1900h. Since no significant differences were found between the GTH levels at 1100h and 1900h within the MT injected group, or within the saline injected group, it seemed that the handling of fish during the three injections disrupted the daily cycle in serum GTH levels on day 7. The fish were exposed to 16L8D/20<sup>0</sup> C for four more days, and the effect of MT on GTH levels was tested again on day 11. One morning injection of MT on day 11 of the 16L8D/20<sup>0</sup> C regime lowered serum GTH levels at 1100h on day 11 in fish which were previously given three injections of MT either in the morning on days 5, 6 and 7 or the afternoons on days 4, 5 and 6. These results indicate either that a single injection of MT in the morning is effective in decreasing serum GTH levels in mid-day in goldfish at this stage of ovarian development and under these environmental conditions, or that the effect of the single morning injection is additive to the effect of the previous three injections. Interestingly, a

significant daily fluctuation in serum GTH levels was detected on day 11 within both the MT and the saline injected groups. No differences in the GSI's or the cellular composition of the ovaries were detected between the saline and the MT injected groups on day 11. The four injections which the fish received during the 11 days of the experiment might not have caused a sufficiently long alteration of the daily cycle in serum GTH levels to influence the gonadal development.

The effect of three morning MT injections on serum GTH levels at 1100h was also investigated in fish subjected to the 16L8D/20<sup>0</sup> C regime for 7 days in late February, 1981; however, no effects were found. It has been demonstrated that an exposure to 16L8D/20<sup>0</sup> for 7 days induces a daily cycle in serum GTH levels in fish in early recrudescence (November), whereas a longer acclimation period is necessary for development of the cycle in fish in the latter stages of ovarian recrudescence (Chapter 1). Possibly, MT alters only the daily pulse in GTH secretion, and an exposure to 16L8D/20<sup>0</sup> C for 7 days in February was not sufficient to induce a well defined daily cycle in serum GTH levels. It may also be that since three morning MT injections inhibit GTH levels in fish in November, but have no effects in fish in February, a seasonal difference in sensitivity of the reproductive axis to MT exists in the goldfish. Data reported by de Vlaming *et al.* (1974a) suggest that the reproductive system of the killifish, *Fundulus similis* exhibits a seasonal variation in sensitivity to MT. Testicular growth was retarded by MT in fish kept under short photoperiod in May, but it had no effect in January. Borg and Ekström (1981) found that while

high doses of MT had antigonadal effects throughout the season in the three-spined stickleback, *Gasterosteus aculeatus*, low doses tended to stimulate ovarian function in winter and summer, but had no effect on the ovary in fall and spring.

Although evidence is accumulating that the pineal organ has a stimulatory influence on the reproductive axis in spring spawning teleosts subjected to long photoperiod and warm temperature conditions in the spring, the understanding of the mechanisms through which the pineal mediates its effects under long photoperiod is limited. It has been proposed by several authors (e.g. Reiter, 1973; Ralph, 1978) that the pineal is a neuroendocrine link conveying photoperiodic information to the hypothalamo-hypophysial axis, and that pinealectomy prevents the animal from responding appropriately to photoperiod changes. In partial support of such a role for the pineal in teleosts, it has been shown that the pineal is in fact a major organizer of circadian activity and has a role in entrainment of circadian rhythms to photoperiodic cues in teleosts (Kavaliers, 1979, 1980, 1981). The evidence that MT had no effect on GTH levels in pinealectomized fish in the present study suggests that MT is not the pineal product that mediates the stimulatory influences of the pineal on the reproductive system under long photoperiod. A similar conclusion was reached by Herbert (1971) who demonstrated that in ferrets, although the pineal has a progonadal influence under long photoperiod, MT did not restore the responsiveness of pinealectomized ferrets to long photoperiod. Secretory products other than MT could be synthesized and secreted by the pineal organ in animals subjected to long photoperiod, and might mediate the stimulatory influences



of the pineal on the reproductive axis. GRH has been identified in the mammalian pineal gland (White *et al.*, 1974; Trentini *et al.*, 1980; Pevet *et al.*, 1980; Piekut and Knigge, 1981; King and Millar, 1981), and seasonal variations in the LHRH content of the pineal have been described in the rat (Joseph, 1976). The presence of GRH in the pineal of teleosts has not been investigated. Alternatively, it has been shown in rainbow trout that some of the nervous pathways originating in the pineal terminate in the nucleus lateralis tuberis region of the hypothalamus (Hafeez and Zerihun, 1975), and since this area is immunoreactive to LHRH (Halpern *et al.*, 1979) and is involved in regulation of gonadal activity, presumably by secretion of the GRH (for review, see Peter (1982)), the pineal could alter the activity of this brain region by its direct afferent input.

Although only the role of the pineal organ in regulation of GTH levels was investigated in experiments discussed in this chapter, the eyes could have also contributed in the mediation of the stimulatory effect of the 16L8D/20<sup>0</sup> C regime. A progonadal influence of the eyes has been detected in the goldfish (Hontela and Peter, 1980; Chapter 3) and the medaka (Urasaki, 1976, 1981) subjected to long photoperiod and warm temperature. Both the eyes and the pineal synthesize MT, and the synthesis is influenced by photoperiod (see Introduction of Chapter 4). Furthermore, the supportive cells in the pineal of the trout have been shown to respond only to photic input from the eyes, whereas the receptor cells in the pineal respond only to photic input through the pineal region (Hafeez *et al.*, 1978). The interaction between the pineal and

the eyes, and their secretory products, and the physiological significance of these interactions remains to be investigated.

The present investigation provides evidence that at certain times of the year, MT, when administered at the appropriate time of day will lower the mid-day peak in serum GTH levels in fish held under the 16L8D/20° C regime. These data conform to numerous reports of the inhibitory effects of MT on GTH levels or gonadal development in various vertebrates, including teleost fishes subjected to long photoperiod and warm temperature (for review, see Reiter (1979, 1980a)). MT administration to goldfish with intact pineal organs held under 16L8D might have a similar effect on the reproductive system as exposure to short photoperiods, which are generally considered to be inhibitory to gonadal development in spring or summer spawning teleosts (for review, see Peter and Crim (1979), Peter (1981)). Endogenous MT is probably released by the teleost pineal in the dark, since MT levels in the blood were higher in mid-scotophase than in mid-photophase (Gern *et al.*, 1978a; Owens *et al.*, 1978). Therefore, the blood levels of MT in fish on a long photoperiod injected with MT early in the light phase might be similar to blood levels of MT in uninjected fish subjected to short photoperiods. Although pineal MT levels, and simultaneous blood or pineal MT levels under various photoperiods have not yet been determined in a teleost, evidence provided by studies in mammals suggests that MT is the, or one of the, pineal antigonadal hormones mediating the influences of short photoperiod or darkness on the reproductive system in long-day breeders. MT levels in the mammalian pineal are influenced by photoperiod and the highest levels are found during darkness (Tamarkin *et*

*al.*, 1979; Rollag *et al.*, 1980; Panke *et al.*, 1980; Reiter *et al.*, 1980; Goldman *et al.*, 1981; Petterborg *et al.*, 1981). Furthermore, blood MT levels are also highest during dark (Rollag *et al.*, 1978; Arendt *et al.*, 1981; Harlow *et al.*, 1981; Lynch *et al.*, 1981).

The present study demonstrates, for the first time in a teleost fish, the existence of a daily rhythm in sensitivity of the reproductive system to MT. However, the mechanisms underlying this rhythm are not understood at present. A daily rhythm in sensitivity to MT has been described in some mammals; the period of sensitivity was detected in the afternoon. It has been proposed that only by the afternoon have the MT receptors on the target organs recovered from the down regulation effect of the nightly MT surge (for review, see Reiter (1980b)). This hypothesis is compatible with observations in rodents that a morning injection of MT prevents the antigonadal effect of MT given in the afternoon (Chen *et al.*, 1980), or that MT continuously available from an implanted capsule counteracts the pineal mediated gonadal regression (Hoffman, 1974; Reiter *et al.*, 1974; Reiter *et al.*, 1975). It seems that in the goldfish, the MT sensitive phase occurs in the morning in female goldfish in early stages of ovarian recrudescence subjected to 16L8D/20<sup>0</sup> C in November. Furthermore, the sensitivity to morning MT injections may disappear later in the year, since morning MT injections had no effect on GTH levels in female goldfish in the latter stages of ovarian development in February. It has not yet been established whether down regulation of MT receptors occurs in the goldfish, and whether the sensitivity to morning MT injections is due to availability of receptors at that time of the day. The factors causing the seasonal

differences in sensitivity to MT are also not known. It has been postulated that in mammals the timing of the MT surge in relation to other circadian activities may determine the effect of MT on the physiology of the animal (Panke *et al.*, 1980). Furthermore, daily cycles in blood (Firth *et al.*, 1979) and pineal (Vivien-Roels *et al.*, 1979) MT levels have been shown to fluctuate seasonally in two species of reptiles. It is conceivable then, that in goldfish held under 16L8D/20° C, MT is secreted into blood in such a cyclic pattern as to allow a response to exogenous MT in the morning. Later in the year, the cycle of MT secretion and possibly of other endocrine parameters might shift in such a way as to cause a loss of sensitivity to a morning pulse of MT. Determination of daily cycles in blood MT levels and of the effect of MT injections at various times of the day in fish held under long or short photoperiod at different times of the year would provide additional data to test this hypothesis.



## GENERAL DISCUSSION

Daily cycles in serum gonadotropin hormone (GTH) levels in female goldfish subjected to various photoperiod and temperature regimes were described for the first time by Hontela and Peter (1978), who also hypothesized that such fluctuating blood GTH levels may be important for stimulation of ovarian development. In the present study, further evidence for the existence of the cycles has been provided, and the role of some exogenous and endogenous factors in regulation of the cycles was investigated. The present study has also provided correlative data that supports the hypothesis about the significance of the daily cycles.

The photoperiod and temperature regimes imposed on female goldfish in the various laboratory experiments were 16 hours of light and 8 hours of dark (16L8D) and 20<sup>0</sup> C, 8L16D/20<sup>0</sup> C, 12L12D/12<sup>0</sup> C, and 16L8D/12-20<sup>0</sup> C diurnal sinusoidal temperature cycles, and an outdoor pond regime in the spring and fall. Under the 16L8D/20<sup>0</sup> C regime, a single peak in serum GTH levels early in the photophase was found in fish in the early stages of ovarian recrudescence ("winter" fish), as well as in fish in the latter stages of ovarian recrudescence ("spring" fish). No significant fluctuations were found under 8L16D/20<sup>0</sup> C in "spring" fish. "Winter" fish subjected to 12L12D/12<sup>0</sup> C had uniform and low serum GTH levels throughout the day, whereas, two peaks in serum GTH levels were found in "spring" fish held under this regime. In all the experiments, the detected patterns of fluctuations in serum GTH levels were similar to the patterns reported previously (Hontela and Peter, 1978),

indicating that the patterns are reproducible under the appropriate conditions.

The persistence of the daily cycles in serum GTH levels was determined for the first time, and seasonal differences in the long term stability of the cycles were found. The peak in serum GTH levels early in the photophase found in fish held under the 16L8D/20<sup>0</sup> C regime was detected as early as days 5 to 7 in fish in early stages of ovarian recrudescence ("winter" fish), but not until days 11 to 13 in fish in the latter stages of ovarian recrudescence ("spring" fish). The peak could no longer be detected on days 11 to 13 in "winter" fish, GTH levels becoming fairly uniform and high throughout the day. The daily fluctuations in serum GTH levels brought about by exposure to 16L8D/20<sup>0</sup> C persist for a longer period in "spring" fish than in "winter" fish. Since the fluctuations seem to stimulate ovarian development, this difference is consistent with the fact that the goldfish is a spring breeder and that exposure to long photoperiod and warm temperature is somehow more physiological in spring than in winter. Differences in the long term stability of the GTH cycles under various photoperiod and temperature regimes were also observed. While significant daily fluctuations in serum GTH levels were still detected in "spring" fish after a 30 day exposure to 12L12D/12<sup>0</sup> C, the mid-day peak was abolished and uniform high GTH levels were found after 30 days of exposure to 16L8D/20<sup>0</sup> C. The data show that photoperiod and temperature, and also the length of the acclimation period to the environmental regime, can influence the patterns of the daily cycles in serum GTH levels.

The similarity of the patterns found in female goldfish held in the outdoor pond to those found in fish held in the laboratory under similar conditions, suggests that cycles in serum GTH levels occur in female goldfish in natural environments. The day-to-day changes in temperature and photoperiod may be of great importance in regulating and maintaining the GTH cycles, and controlling the rate of ovarian development. Based on the results of this study and data reported by Hontela and Peter (1978), it may be predicted that low and uniform serum GTH levels would occur in female goldfish undergoing the early stages of ovarian recrudescence in the fall and early winter. Under the increasing daylength and relatively cold temperatures of early spring, fish in the latter stages of ovarian recrudescence would have cycles similar to those detected under the 16L8D/12<sup>0</sup> C regime in the laboratory or in the outdoor pond in April (1981 experiment). Fish in the final stages of ovarian development (late spring) would have large fluctuations in serum GTH levels similar to those described for fish under 16L8D/20<sup>0</sup> C in the laboratory.

Serum GTH levels may be influenced by the secretory rate from the pituitary and the metabolic clearance rate of GTH from blood. These parameters are diagrammatically represented in Figure 5.1. It was attempted to determine whether changes in the pituitary GTH content were correlated with fluctuations in serum GTH levels. A significant daily variation in pituitary GTH levels was found only in one group of fish in which a relatively large and sharp peak in serum GTH levels was detected (group held under 16L8D/20<sup>0</sup> C for 5 to 7 days in November); however, a significant negative correlation between the daily cycles

Figure 5.1. A tentative model representing diagrammatically some of the hypotheses formulated from the experimental data in the present study.

Explanation for some of the symbols used:

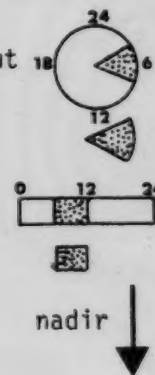
responsiveness to a stimulus throughout  
24 hours

greatest responsiveness

secretion rate throughout 24 hours

largest rate

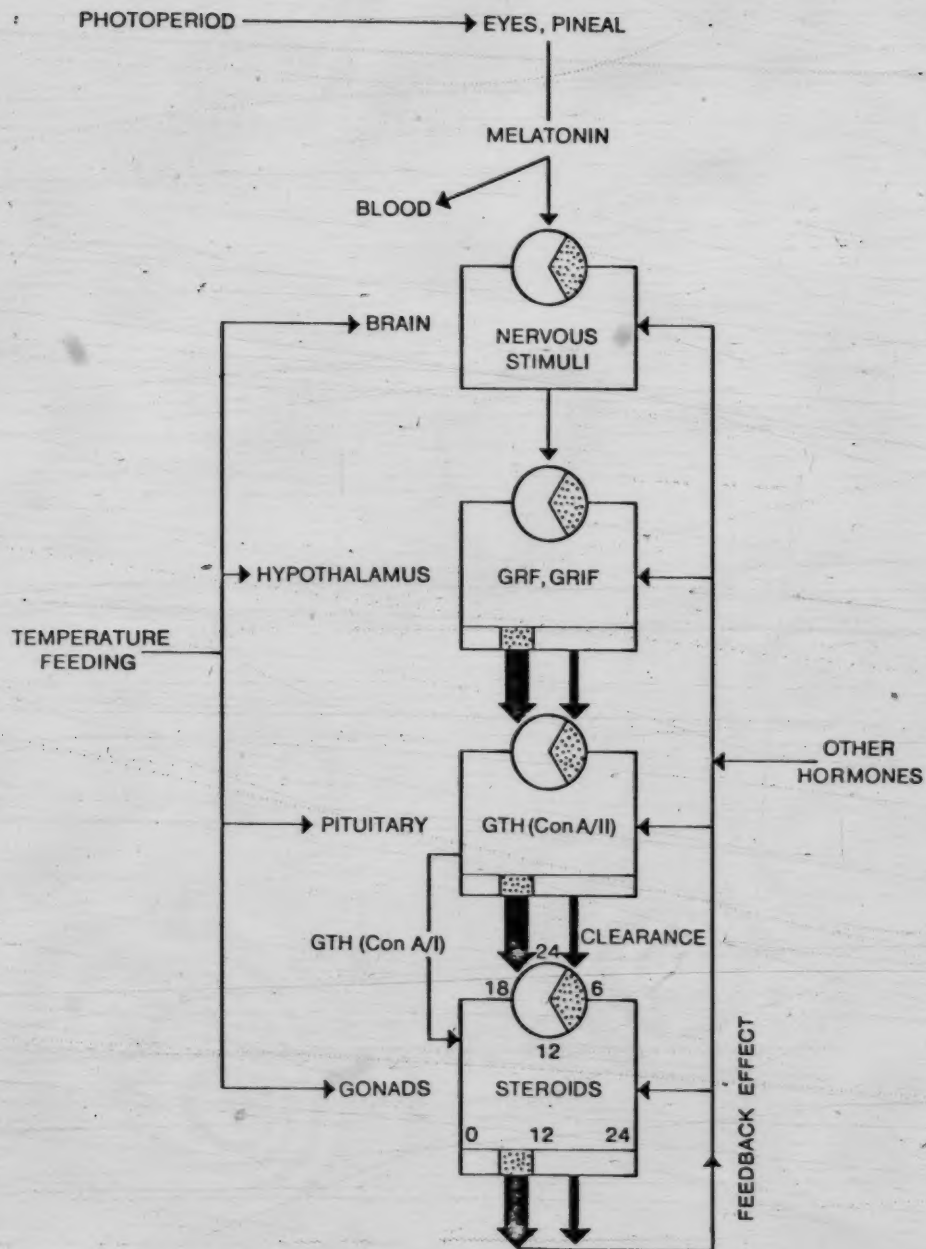
levels in blood      apogee



nadir







in serum and pituitary GTH levels was not detected. When the hourly pituitary secretion rate of GTH was calculated using the estimated metabolic clearance rate of GTH in female goldfish under similar environmental conditions (Cook and Peter, 1979a), and the serum GTH levels measured in this study, it was found that the amount of GTH released from the pituitary is a small proportion of the amount stored. It has been reported that temperature and the stage of ovarian development influence the metabolic clearance rate of GTH (Cook and Peter, 1979a) and ovarian uptake of GTH (Cook and Peter, 1979b) in female goldfish. However, whether daily changes in the metabolic clearance rate and rate of ovarian uptake of GTH occur is not known; perhaps factors such as these, in addition to the pituitary secretion rate, could have influences on the daily cycles in serum GTH levels.

The present study provided further evidence that a daily cycle in serum GTH levels is more stimulatory to ovarian development in goldfish than steady low or high levels. The peak in serum GTH levels usually found in fish kept under the 16L8D/20<sup>0</sup> C regime was abolished by an extended exposure to this regime (Chapters 1 and 2), exposure to warm temperature only during night (Chapter 2), and pinealectomy and/or blinding (Chapter 3). In these experiments the disappearance of the peak was correlated with lower GSI's and/or severe atresia of the ovaries, compared to fish with fluctuating serum GTH levels in the same experiments. Two experiments did not positively support the hypothesis that daily cycles in serum GTH levels are important for stimulation of ovarian development; inconclusive evidence was provided by the light and feeding shift experiment (Chapter 2), and a melatonin (MT) injection

experiment (Chapter 4) in which the mid-day serum GTH levels were decreased by the treatment but there were no effects on ovarian condition, as judged by histology. In this latter experiment, possibly the number of injections and therefore the number of days during which the GTH levels were altered was not sufficient to have an effect on the gonads.

Future experiments should be aimed at understanding of the mechanisms by which the fluctuating serum GTH levels promote ovarian development in teleosts. The desensitizing effect of constant high serum GTH levels on the ovary has been well documented in mammals and is interpreted to be the result of down regulation of ovarian GTH receptors (for review, see Catt *et al.* (1979)). These receptors were characterized and manipulated by various treatments in mammals (e.g. O'Shaughnessy and Brown, 1978; Chan *et al.*, 1981), as well as birds (Ishii and Farner, 1976; Tsutsui and Ishii, 1978), reptiles (Licht *et al.*, 1977) and amphibians (Kubokawa and Ishii, 1980). Furthermore, the responsiveness of the ovary to GTH has been shown to vary throughout the day in some tetrapods (Lamond and Braden, 1959; Dusseau and Bosher, 1976; Grizzard *et al.*, 1978; Easley *et al.*, 1979; Nankin *et al.*, 1980), possibly because of fluctuations in the number and/or affinity of the ovarian GTH receptors. Although the receptors for GTH have not been characterized in teleost fishes, daily changes in responsiveness to exogenous GTH have been shown in the ovary of the shiner (de Vlaming and Vodcnik, 1977), rainbow trout (Kuo and Watanabe, 1978) and goldfish (Peter *et al.*, 1982). In goldfish subjected to a 16L8D/14<sup>0</sup> C regime, the period of greatest responsiveness coincided with the timing of the peaks in serum GTH levels in female goldfish held under similar

environmental conditions (Hontela and Peter, 1978). This suggests that the time of day during which GTH is available to the ovary, rather than the overall amount of GTH available throughout the day, determines the extent of stimulation of ovarian development. The daily changes in responsiveness of the ovary to GTH, and possibly also to other hormones, are represented in Figure 5.1. While constant low serum GTH levels may maintain a relatively slow rate of oocyte growth, particularly during low temperature regimes in mid-winter, the growth is greatly stimulated when there are daily peaks in serum GTH levels. When the timing of such peaks coincides with the daily period of ovarian responsiveness, ovarian growth is optimized. Constant high serum GTH levels desensitize the ovary and oocyte atresia ensues. This hypothesis could in part be tested by characterizing the ovarian GTH receptors in female goldfish exposed to environmental conditions similar to those used in the present study.

Other hormones could act synergistically with GTH on the ovary, and influence or regulate the daily cycles in GTH levels (see Figure 5.1). In mammals gonadal steroids have been shown to influence ovarian receptors for LH and FSH (Richards *et al.*, 1979; Farookhi, 1980) and to modify pituitary sensitivity to luteinizing hormone-releasing hormone (LHRH) (Clayton *et al.*, 1980; Geiger *et al.*, 1980; Kesner *et al.*, 1981). That this could also occur on diurnal basis is suggested by reports that plasma levels of sex steroids fluctuate throughout the day in mammals (Wilson *et al.*, 1976; Glowania *et al.*, 1979; Miyatake *et al.*, 1980). The role of sex steroids and gonadotropin releasing hormone (GRH) in the regulation of the hypothalamo-hypophysial-gonadal axis in teleosts is



well documented (for review, see Peter (1982)), but daily cycles in the blood levels of steroids and daily cycles in responsiveness to GRH or gonadotropin release-inhibitory factor (GRIF) remain to be shown. Also, it remains to be demonstrated whether a second GTH (Con A-I/GTH, see General Introduction) exists in the goldfish, and has a role in the regulation of ovarian development, possibly on a diurnal basis. However, daily fluctuations in blood levels of cortisol (Fryer, 1975; Delahunty and de Vlaming, 1980; Delahunty *et al.*, 1978b; Peter *et al.*, 1978b), prolactin (Leatherland and McKeown, 1973; McKeown and Peter, 1976; Spieler *et al.*, 1976), thyroid hormones (White and Henderson, 1977; Osborne *et al.*, 1978; Spieler and Noeske, 1979, 1981; Easles *et al.*, 1981) and growth hormone (Leatherland *et al.*, 1974) have been demonstrated in teleosts, and daily fluctuations in these hormones may also influence the activity of the teleost reproductive system at its various levels. Interestingly, daily fluctuations in brain tissue levels of catecholamines (Sauerbeir and Meyer, 1977) and serotonin (Fingerman, 1976; Genot *et al.*, 1981) have also been reported in some teleosts.

The role of some environmental cues in regulation of the daily cycles in serum GTH levels was investigated. It appears that the timing of diurnal temperature fluctuations, the time of day of feedings, and the photoperiod interact to influence the patterns of the GTH cycles. The mechanisms involved in photoperiod input have been investigated to some extent in the present work (Chapters 3 and 4). However, the route by which temperature and feeding mediate their effects on the reproductive system has not been investigated. Perhaps the central nervous system has a daily rhythm in responsiveness to temperature and

feeding, as depicted in Figure 5.1. Feeding times, temperature and photoperiod have been shown to modify the daily cycles in levels of some carbohydrates and lipids in blood and liver of goldfish (Delahunty *et al.*, 1978b; Delahunty and de Vlaming, 1980). Temperature and feeding may therefore influence the availability and the rate of utilization of various metabolites necessary for the synthesis of hormones, or for ovarian development. Although this may be part of the mechanism for inducing daily cyclicity in responsiveness of the ovary to GTH, what induces such variations in responsiveness is not known. Whether this may also influence the responsiveness of the brain to various inputs is an open question.

Photoperiod has been shown to have an effect on daily cycles in serum GTH levels (Hontela and Peter, 1978). In the present study, some of the mechanisms through which photoperiod influences GTH secretion were investigated. The pineal, and possibly also the eyes, promote daily fluctuations in serum GTH levels under long photoperiod in the spring, whereas only the pineal has been shown to inhibit these fluctuations under short photoperiod. On the other hand, pinealectomy had no effect on serum GTH levels in the fall in sexually regressed fish (Chapter 3), and presumably has no influence on the reproductive axis in such fish. The pineal gland therefore seems to mediate the effects of photoperiod on the gonads by altering the patterns of the daily cycles in serum GTH levels.

Melatonin (MT) injections lowered the early-photophase peak in serum GTH levels in intact female goldfish in early stages of ovarian recrudescence (November) under long photoperiod and warm temperature

conditions. Since MT is presumably synthesized in the pineal during the dark (Fenwick, 1970b; Gern *et al.*, 1978a), this evidence indicates that MT may be the pineal antigonadal compound in the goldfish, and that it mediates the inhibitory photoperiodic influences on the reproductive system. A daily rhythm in sensitivity of the hypothalamo-hypophysial axis to MT injections has been demonstrated; only morning injections were effective in lowering the serum GTH levels. MT has been detected in the blood of teleosts (Gern *et al.*, 1978a; Owens *et al.*, 1978), therefore it presumably can directly influence the target organs within the reproductive axis. In mammals, MT has been reported to have effects on the GRH secretion (Kao and Weisz, 1977) from isolated hypothalamus, to inhibit the pituitary response to LHRH (Martin *et al.*, 1977, 1980; Bacon *et al.*, 1981) *in vivo* and *in vitro*, and to influence steroid production by the gonads *in vitro* (Younglai, 1979; MacPhee *et al.*, 1975; Kano and Miyachi, 1976). Furthermore, a nervous pathway has been demonstrated between the pineal and the hypothalamus of the rainbow trout (Hafeez and Zerihun, 1975), and MT could therefore also act as a neurotransmitter directly on the brain. The compound responsible for the progonadal effect of the pineal under long photoperiod and warm temperature is not known.

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## APPENDIX 1

## Effects of Pinealectomy, Blinding, and Sexual Condition on Serum Gonadotropin Levels in the Goldfish

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The effects of pinealectomy and/or blinding on serum gonadotropin (GTH) levels and gonadal development were investigated in sexually regressed male and female goldfish subjected to long photoperiod and warm temperature, and in mature female fish subjected to either long or short photoperiod and warm temperature. Control (intact and/or sham operated), pinealectomised, blind-pinealectomised, and blinded fish were blood sampled at 12 and 20 hr on Day 7, 8, or 9 of the experimental photoperiod-temperature regime. Serum GTH levels were determined by radioimmunoassay. Pinealectomy had no effect while blinding promoted a daily cycle in serum GTH levels in regressed fish subjected to 16L:8D/21° in November; no effect on gonadal development was found under this regime. Pinealectomy, and possibly also blinding, repressed a daily cycle in serum GTH levels found in control fish, and caused a decrease in gonadal development, in mature female fish under 16L:8D/21° in the spring. No daily cycle in serum GTH levels was detected in control or in blinded mature female fish under 8L:16D/21° in the spring. Pinealectomy promoted a daily cycle in serum GTH, but no effects of pinealectomy or blinding on gonadal development were detected, under this regime. The results indicate that the pineal influences gonadal development through, at least in part, alteration of the daily cycles of serum GTH levels in goldfish.

The presence of photoreceptor-like elements in the teleost pineal organ has been demonstrated by means of electron microscopy in several species (e.g., Bergmann, 1971; Oguri and Omura, 1973), including the goldfish, *Carassius auratus* (Takahashi, 1969). Electrophysiological studies (Dodt, 1963; Morita and Bergmann, 1971; Hanyu *et al.*, 1978) indicate that the neuronal discharge activity of the teleost pineal increases as light intensity decreases. Furthermore, the biochemical activity of the pineal organ of teleosts fluctuates throughout the day and is influenced by light-dark cycles. In the pineal of rainbow trout, *Salmo gairdneri*, melatonin levels are highest during the scotophase (Gern *et al.*, 1978; Owens *et al.*, 1978), and the activity of hydroxyindole-O-methyl transferase (HIOMT), the enzyme which converts serotonin to melatonin, is also highest during the scotophase (Smith and Weber, 1976).

It has been assumed that the pineal of teleosts is independent of the retina, in contrast to the pineal gland of mammals which is sympathetically innervated and receives photic input from the eyes (Wurtman *et al.*, 1968). However, Fenwick (1970a) showed that the phototactic response of the goldfish depended upon the presence of both the pineal and the eyes. Furthermore, Weber and Smith (1976) found that only blinding abolished photoperiod-induced HIOMT cycles in rainbow trout, while preventing direct illumination of the pineal alone had no effect on these cycles.

The pineal of teleosts has been linked to several physiological processes including reproduction (for review see Wurtman *et al.*, 1968; Relkin, 1976; Reiter, 1977, 1978). Pinealectomy caused gonadal regression in recrudescing or sexually mature shiners, *Notemigonus crysoleucas* (de Vlaming, 1975), or goldfish (de Vlaming and Vodicnik, 1978) under long photoperiod and



warm temperature, and in recrudescing shiners under long photoperiod and cold temperature (de Vlaming, 1975). A similar effect of pinealectomy and/or blinding was also observed in the medaka, *Oryzias latipes*, held under long photoperiod and 18° (Urasaki, 1972, 1973, 1976). No effects of pinealectomy were found in shiners held at a cold (12°) temperature and short photoperiod (de Vlaming, 1975). On the other hand, pinealectomy accelerated gonadal development in goldfish maintained on short photoperiod and 13° (Fenwick, 1970b) or 20° (de Vlaming and Vodcnik, 1978) in the spring. A similar stimulation of gonadal development following pinealectomy was also found in the shiner (de Vlaming, 1975) and in the medaka (Urasaki, 1973, 1976) under short photoperiod and warm temperature. Thus, in fishes that respond by gonadal recrudescence to increasing daylength and long photoperiods in the spring (see review by de Vlaming, 1972; Peter and Crim, 1979), the pineal, and probably also the eyes, has a role in the stimulation of gonadal recrudescence under long photoperiods, and in the suppression of gonadal activity under short photoperiods.

Vodcnik *et al.* (1978) have reported that the effects of pinealectomy on pituitary and plasma gonadotropin (GTH) levels in goldfish may depend on the photoperiod-temperature regime and stage of sexual development. The daily cycles of serum GTH levels in the goldfish, and the effects of photoperiod, temperature, and sexual condition on these cycles, have been described in detail (Hontela and Peter, 1978). Since goldfish undergoing ovarian recrudescence and sexually mature females generally exhibit significant fluctuations in serum GTH levels, whereas sexually regressed fish do not (Hontela and Peter, 1978), we have attempted to determine the effects of pinealectomy and blinding on serum GTH levels in goldfish at two different times of day and at different stages of ovarian recrudescence and under different environmental regimes.

## MATERIALS AND METHODS

### Experimental Animals

Goldfish (common or comet variety, standard body length 6.5–8.0 cm) were purchased from Grassyfork Fisheries Company Inc., Martinsville, Indiana. In November, male and female fish, which were sexually regressed or in early gonadal recrudescence, were selected for the experiment since it was not possible to externally sex the fish. In experiments in March and April, females with ovaries containing at least some oocytes that had completed vitellogenesis ("mature" females) were selected.

### Photoperiod and Temperature

A summary of the protocol is presented in Fig. 1. After arrival from the fish farm, fish were held in a 4800-liter flow-through aquarium under a simulated natural photoperiod (Edmonton) and 12–15° for about 14 days. They were then transferred into three 380-liter flow-through aquaria maintained under 12 hr light and 12 hr dark (12L:12D, lights on at 0800 hr) and 12° for 8 days, each aquarium containing 40 fish. During Days 7 and 8 of this regime the fish in each aquarium were divided into experimental groups and surgery (see Operations) performed. Postoperatively the fish were kept under 12L:12D and 12° for 8 more days, and finally the experimental regimes of 16L:8D/21° or 8L:16D/21° were imposed (designated as Day 1 on Fig. 1) for a maximum of 9 days. The temperature of 21° was obtained by raising the temperature over a 12-hr period, starting in the morning of Day 1. Fish were fed *ad libitum* at about 1 and 7 hr after lights on.

### Blood Samples

A presample of blood was taken from all the fish on Day 4 of the experimental regime (see Fig. 1). Subsequent samples were taken at 1200 and 2000 hr on Day 7, 8, or 9. These sampling times were chosen because in our previous studies (Hontela and Peter, 1978) an apogee in serum GTH levels was detected at 1200 hr and a nadir at 2000 hr in sexually maturing or mature female goldfish exposed to 16L:8D/21°. At each sampling time, at 15 min to the hour 10 fish were caught and anaesthetized in a 1:1000 solution of tricaine methanesulphonate and blood sampled, and on the hour the procedure was repeated with 10 more fish. Blood was withdrawn from the caudal vasculature and serum collected as described by Hontela and Peter (1978). After blood sampling on Day 7, 8, or 9 the fish were killed, and total body weight and gonad weight were determined for calculation of the gonadosomatic index (GSI).

### Gonadotropin Radioimmunoassay

Serum GTH levels were determined by radioimmunoassay (RIA) using purified carp GTH (cGTH) as standards and antibody to cGTH (Crim *et al.*, 1976; Hontela and Peter, 1978). The cGTH used in the assays (gift from B. Breton) was purified by a scheme

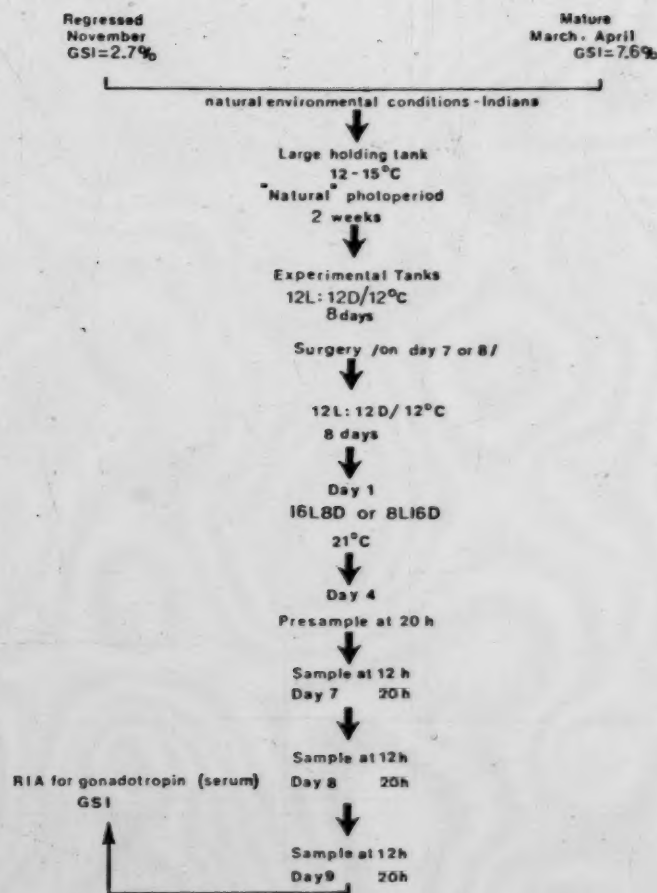


FIG. 1. An outline of the protocol for the experiments.

utilizing, in part, adsorption of glycoprotein cGTH on concanavalin A-Sepharose and elution with buffer containing 0.15 M 1-O-methyl- $\alpha$ -D-mannopyranoside (B. Breton, personal communication). For RIA, the purified glycoprotein cGTH was labelled with  $^{125}\text{I}$  by the standard chloramine-T method. After initial separation of  $^{125}\text{I}$ -cGTH from inorganic  $^{125}\text{I}$  by chromatography on a Sephadex G-50 (fine)  $1 \times 10$ -cm column, the glycoprotein  $^{125}\text{I}$ -cGTH was separated from nonglycoprotein fragments of  $^{125}\text{I}$ -cGTH by adsorption on concanavalin A-Sepharose, and the nonglycoprotein material eluted with barbital buffer pH 8.6, followed by elution of the glycoprotein cGTH with barbital buffer containing 0.15 M 1-O-methyl- $\alpha$ -D-mannopyranoside. Thus, the GTH RIA done here measured the "Con AII" glycoprotein GTH, similar to that described by Ng and Idler (1978a, b). The performance characteristics of the RIA were as described

previously (Crim *et al.*, 1976; Hontela and Peter, 1978).

### Operations

Fish were anaesthetised and wrapped in a wet paper towel.

**Blinding.** The conjunctiva membrane was grasped with fine notched tweezers and cut entirely around the eyeball. The membranes remaining on the posterior of the eyeball were grasped and the eyeball was slightly pulled anteriorly and out. Blunt curved scissors were then inserted from the posterior side of the eye, passing over the blood vessel which is situated on the posterior side of the orbit, to cut the optic nerve and the eye muscles, allowing the removal of the eyeball. This procedure caused very little bleeding. The blinded fish were observed to resume feeding about 2 days postoperatively.

**Pinelectomy.** The forebrain was exposed, as described by Peter (1970) and Peter and Gill (1975), and the *saccus dorsalis* and the pineal stalk, visible anterior to the optic lobes under a dissecting microscope (24 $\times$ ), were removed by suction with a Pasteur pipet. The operation was considered successful when the two structures were observed to move into the pipet and when slight bleeding appeared at the site where the *saccus dorsalis* attaches to the brain. In order to remove the pineal body, gently suction was applied to the inner surface of the cranium in the region anterior to the exposed area (Fig. 2). The cranium was then filled with physiological saline and closed using the procedure described by Peter (1970) and Peter and Gill (1975).

**Sham operation.** All the steps described for pinelectomy were carried out except that the *saccus dorsalis*, the pineal stalk, and pineal body were not removed. A sham operation for blinding was not done.

#### Statistical Analysis

There were no significant differences, as indicated by an analysis of variance (ANOVA), in the serum GTH levels between groups of fish from an experi-

mental group sampled at 1200 or 2000 hr over Days 7, 8, and 9. The values for fish within an experimental group at either 1200 or 2000 hr on Days 7, 8, and 9 were therefore pooled for statistical comparison by ANOVA and the Mann-Whitney *U* test. Both two-tailed and one-tailed *U* tests at  $P < 0.05$  were used and are referred to in the results as ( $P < 0.05$ ) and ( $P < 0.05$ , one tailed), respectively. A two-tailed Student's *t* test at  $P < 0.05$  was used to determine significant differences between the GSI values.

#### RESULTS

The serum GTH levels of sexually regressed-early recrudescence goldfish exposed to 16L:8D/21° in November are shown in Fig. 3. The values at 1200 and 2000 hr in both intact and sham-operated control fish were not significantly different, similar to the values reported by Hontela and Peter (1978) for fish at a similar sexual state under these conditions. However, the values of the blind group at 1200 hr were

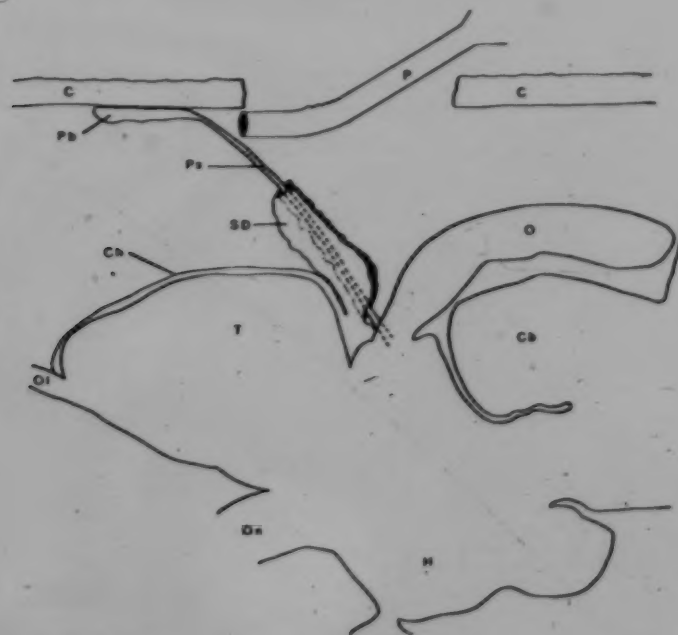


FIG. 2. Diagrammatic representation of sagittal section through the goldfish forebrain showing the pineal stalk (Ps) enveloped by folds of the *saccus dorsalis* (SD) and the orientation of a suction pipet (P) used to remove the pineal body (Pb) from under the roof of the cranium (C). Other abbreviations: Cb, cerebellum; Ch, choroid membrane; H, hypothalamus; O, optic tectum; Ol, olfactory tract; On, optic nerve; T, telencephalon.

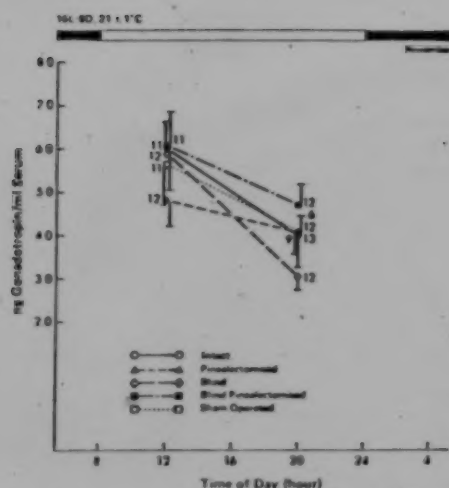


FIG. 3. Serum GTH levels (mean  $\pm$  SEM) of intact, sham-operated, blind, and/or pinealectomized sexually regressed goldfish subjected to 16L:8D/21° in November. The numbers beside each point represent number of fish sampled at each time. The black horizontal bar represents the dark phase of the photoperiod and the empty bar represents the light phase. Statistically significant differences: 1200 hr blind > 2000 hrs blind ( $P < 0.05$ ).

significantly higher than the values of the blind group at 2000 hr ( $P < 0.05$ ). Males were included in the November experiment only; exclusion of the males did not significantly alter the data, and males seem to have similar daily cycles in serum GTH levels compared to females (A. Hontela and R. E. Peter, unpublished).

The serum GTH levels in mature female goldfish exposed to 16L:8D/21° in early March are shown in Fig. 4. The GTH levels of the intact group at 1200 hr were significantly higher than the values of the intact group at 2000 hr ( $P < 0.05$ ), similar to the values reported by Hontela and Peter (1978) for mature female fish under these conditions. Also, the values of the sham-operated group at 1200 hr were higher than the values of the sham-operated group at 2000 hr ( $P < 0.05$ , one tailed); there were no differences between the intact and sham groups at either 1200 or at 2000 hr. The values of the intact group at 1200 hr were sig-

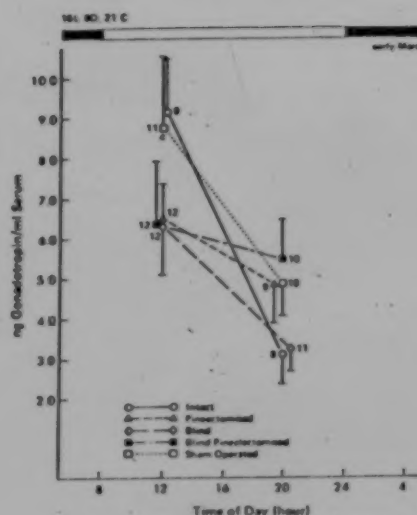


FIG. 4. Serum GTH levels (mean  $\pm$  SEM) of intact, sham-operated, blind and/or pinealectomized mature female goldfish subjected to 16L:8D/21° in early March. (See legend to Fig. 3 for more detail.) Statistically significant differences: 1200 hr intact > 2000 hr intact ( $P < 0.05$ ), 1200 hr intact > 1200 hr blind-pinealectomized ( $P < 0.05$ ), 1200 hr intact > 1200 hr blind ( $P < 0.05$ , one tailed), 1200 hr intact > 1200 hr pinealectomized ( $P < 0.1$ , one tailed); 1200 hr sham > 2000 hr sham ( $P < 0.05$ , one tailed); 1200 hr blind > 2000 hr blind ( $P < 0.05$ ).

nificantly higher than the values of the blind-pinealectomized group at 1200 hr ( $P < 0.05$ ), and the blind group at 1200 hr ( $P < 0.05$ , one tailed), and tended to be higher than the pinealectomized group at 1200 hr ( $P < 0.10$ , one tailed). The values of the blind group at 1200 hr were significantly higher than the values of the blind group at 2000 hr ( $P < 0.05$ ).

Serum GTH levels in fish exposed to 16L:8D/21° in late April are shown in Fig. 5. The values of the intact group at 1200 hr were higher than the values of the intact group at 2000 hr ( $P < 0.05$ , one tailed), and the values of the sham-operated group at 1200 hr were higher than the values of the sham-operated group at 2000 hr ( $P < 0.05$ , one tailed). Also, the serum GTH levels of the intact group at 1200 hr were higher than the serum levels of the pinealectomized and



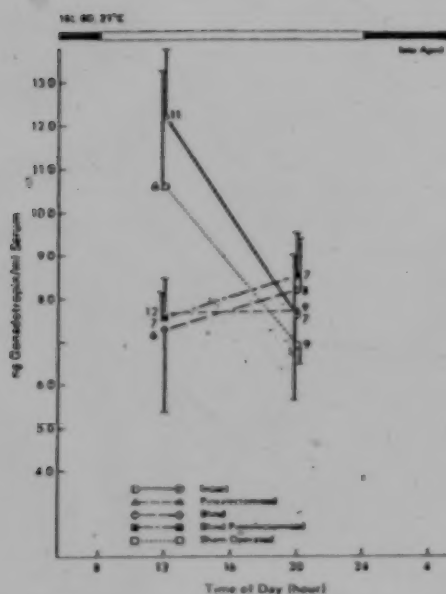


FIG. 5. Serum GTH levels (mean  $\pm$  SEM) of intact, sham-operated, blind, and/or pinealectomized mature female goldfish subjected to 16L:8D/21° in late April. (See legend to Fig. 3 for more detail.) Statistically significant differences: 1200 hr intact > 2000 hr intact ( $P < 0.05$ , one tailed), 1200 hr intact > 1200 hr pinealectomized ( $P < 0.05$ ), 1200 hr intact > 1200 hr blind-pinealectomized ( $P < 0.05$ ); 1200 hr sham > 2000 hr sham ( $P < 0.05$ , one tailed).

blind-pinealectomized groups at 1200 hr ( $P < 0.05$ ).

The serum GTH levels of mature female fish subjected to 8L:16D/21° in mid-March are shown in Fig. 6. The 1200- and 2000-hr values of the sham control group were not significantly different, similarly to previous results for mature fish under these conditions (Hontela and Peter, 1978). (An intact group was not included in this experiment.) The values of the pinealectomized group at 1200 hr were higher than the values of the pinealectomized group at 2000 hr ( $P < 0.05$ ). Also, the values of the blind-pinealectomized group at 1200 were higher than the values of the blind-pinealectomized group at 2000 hr ( $P < 0.05$ , one tailed). The serum GTH levels at 1200 and 2000 hr were not significantly different in the blind group.

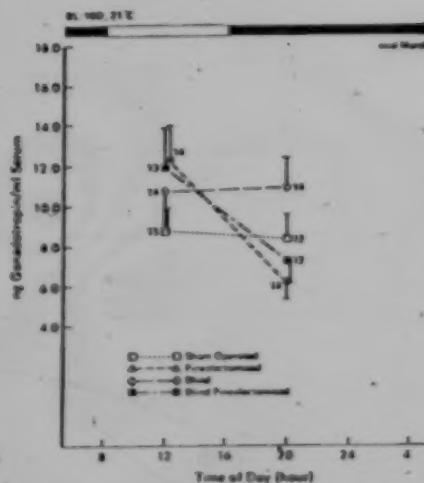


FIG. 6. Serum GTH levels (mean  $\pm$  SEM) of sham-operated, blind, and/or pinealectomized mature female fish subjected to 8L:16D/21° in mid-March. (See legend to Fig. 3 for more detail.) Statistically significant differences: 1200 hr pinealectomized > 2000 hr pinealectomized ( $P < 0.05$ ); 1200 hr blind-pinealectomized > 2000 hr blind-pinealectomized ( $P < 0.05$ , one tailed).

The presample values (2000 hr on Day 4) were not significantly different from grouped 2000-hr values on Days 7, 8, and 9 in 16 out of the 19 experimental groups; the presample values were lower ( $P < 0.05$ ) than the 2000-hr values on Days 7, 8, and 9 in the 16L:8D/21° sham-operated groups from March and April, and in the 16L:8D/21° blind-pinealectomized group from March.

The GSIs of the various experimental groups are presented in Table 1. There were no significant differences between the groups in the November experiment. In the groups exposed to 16L:8D/21° in March, the GSI of the intact group was higher than the GSI of the blind-pinealectomized group ( $P < 0.05$ ), and the GSI of the sham-operated group was higher than the GSI of the blind-pinealectomized, and the pinealectomized groups ( $P < 0.05$ ), respectively. The GSI of the intact group subjected to 16L:8D/21° in April was higher than the GSI of the blind and the pinealectomized groups.

TABLE 1  
EFFECTS OF PINEALECTOMY AND BLINDING ON THE GONADOSOMATIC INDEX OF GOLDFISH

| Photoperiod-<br>temperature<br>regime | Time<br>of<br>year | Gonadosomatic index ( $\bar{X} \pm \text{SEM}$ ) |                         |                                      |                                        |                                      |
|---------------------------------------|--------------------|--------------------------------------------------|-------------------------|--------------------------------------|----------------------------------------|--------------------------------------|
|                                       |                    | Intact                                           | Sham<br>operated        | Blind                                | Blind<br>pinealectomised               | Pinealectomised                      |
| 16L:8D/21°                            | November           | 2.8 $\pm$ 0.3<br>N = 20                          | 3.0 $\pm$ 0.2<br>N = 23 | 2.7 $\pm$ 0.2<br>N = 24              | 2.8 $\pm$ 0.2<br>N = 23                | 2.3 $\pm$ 0.8<br>N = 25              |
| 16L:8D/21°                            | Early<br>March     | 7.4 $\pm$ 0.9<br>N = 17                          | 7.6 $\pm$ 0.7<br>N = 21 | 6.5 $\pm$ 0.5<br>N = 23              | 5.0 $\pm$ 0.5 <sup>a,b</sup><br>N = 23 | 5.9 $\pm$ 0.6 <sup>a</sup><br>N = 21 |
| 16L:8D/21°                            | Late<br>April      | 10.6 $\pm$ 1.2<br>N = 16                         | 8.6 $\pm$ 1.3<br>N = 15 | 7.0 $\pm$ 0.9 <sup>a</sup><br>N = 14 | 9.3 $\pm$ 1.4<br>N = 14                | 7.1 $\pm$ 1.1 <sup>a</sup><br>N = 21 |
| 8L:16D/21°                            | Mid<br>March       | —                                                | 8.5 $\pm$ 0.7<br>N = 27 | 7.6 $\pm$ 0.8<br>N = 28              | 7.7 $\pm$ 0.7<br>N = 25                | 7.3 $\pm$ 0.8<br>N = 28              |

<sup>a</sup> Significantly lower ( $P < 0.05$ ) than intact group in the same experiment.

<sup>b</sup> Significant lower ( $P < 0.05$ ) than sham-operated group in the same experiment.

tomised groups ( $P < 0.05$ ) in that experiment.

#### DISCUSSION

The experimental design of the present study is similar to that used previously to demonstrate the effects of photoperiod, temperature, and sexual condition on the daily cycles in serum GTH levels in the female goldfish (Hontela and Peter, 1978). Previously an apogee and a nadir in serum GTH levels were found at 1200 and 2000 hr (4 and 12 hr after lights on), respectively, in sexually mature female goldfish subjected to 16L:8D/21° in the spring. Because of these findings, 1200 and 2000 hr were chosen as the two sampling times in the present study. Similar to our previous results, the 1200-hr GTH levels were higher than the 2000-hr levels in intact, and also in sham-operated mature females under 16L:8D/21° in the spring. In addition, similar to patterns described by Hontela and Peter (1978) for fish under these conditions, the 1200- and 2000-hr serum GTH levels were not significantly different in mature females under 8L:16D/21° in the spring, and in regressed fish under 16L:8D/21° in the fall. Therefore, it is assumed that the daily cycles of serum GTH in the control groups in the present study are similar to the cycles found previously, and that the effects of pinealectomy

and blinding on serum GTH levels at 1200 and 2000 hr may reflect changes in the daily cycle. This is further supported by the pre-sample values taken at 2000 hr on Day 4; the presample values within experimental groups were generally similar to those obtained at 2000 hr on Days 7, 8 and 9. Since the nadir of the daily cycle was previously detected at 2000 hr, this evidence suggests that at least that part of the cycle is stable.

Since the daily cycle of serum GTH can vary with the state of gonadal development in goldfish (Hontela and Peter, 1978), the blood samples in the present experiments were taken relatively soon postoperatively so that the state of the gonads would not be greatly altered before sampling. Postoperatively the fish were exposed to 12L:12D/12° for 8 days; no effects of pinealectomy were found in goldfish held at 12L:12D/24.5° (Peter, 1968), and changes in gonadal state are slow in goldfish at 12° compared to 21° (R. Peter and A. Hontela, unpublished). Exposure to the experimental conditions of 16L:8D/21° or 8L:16D/21° for next 7 to 9 days is a relatively short period compared to length of time after which pinealectomy and/or blinding were found to have significant effects on the reproductive system in other investigations (21 to 28 days, de Vlaming and Vodienik, 1978; 45 to 115 days, Fenwick, 1970b; 37 days, Urasaki, 1973; 22

days, Vodicnik *et al.*, 1978). In spite of the relatively short exposure to experimental conditions in our study, some significant effects of pinealectomy and/or blinding on GSI were found. However, since the changes did not represent major differences in the state of the gonads (e.g., change from mature to regressed condition), it is likely that the differences in serum GTH levels were due to the operations and not to changes in gonadal condition.

The results indicate that the effects of pinealectomy and blinding on serum GTH levels and on gonadal development in the goldfish depend on the photoperiod-temperature regime to which the fish are exposed, and on the stage of sexual development of the fish. In November, pinealectomy has no effect on either serum GTH levels at 12 and 20 hr, or the GSI of fish exposed to a long photoperiod and warm temperature. Similar evidence, although based on samples with smaller numbers of fish, has been provided by Vodicnik *et al.* (1978) who found no effect of pinealectomy on pituitary and serum GTH levels throughout a 24-hr period in female goldfish held at 15.5L:8.5D/24° in November. Fenwick (1970b) also found no effect of pinealectomy on GSI in goldfish exposed to 16L:8D/21° from October to December. It has been suggested previously (Hontela and Peter, 1978) that the hypothalamo-pituitary-gonadal axis in goldfish in September is relatively unresponsive to long photoperiod and warm temperature, compared to fish in January or March. Our results suggest that the reproductive system of sexually regressed-early recrudescence fish held under 16L:8D/21° in November is similarly unresponsive to pinealectomy. Interestingly, only blinded fish with intact pineal showed significant differences in serum GTH levels between 1200 and 2000 hr, indicating that a daily cycle in serum GTH levels was promoted by blinding. The mechanism for this is not known but perhaps in sexually regressed-early re-

crudescence female fish the eyes may suppress rhythmicity whereas the pineal may stimulate rhythmicity independent of influence of eyes. Further investigations are required to clarify this.

In the two spring experiments on sexually mature females under 16L:8D/21°, pinealectomy, and blinding and pinealectomy abolished the significant difference in serum GTH levels between 1200 and 2000 hr found in the control groups and, compared to the intact controls, significantly lowered the levels at 1200 hr so that the values were similar to the 2000-hr values. Blinding, on the other hand, abolished the significant difference in serum GTH levels between 1200 and 2000 hr in only one experiment (April), and significantly reduced the 1200-hr levels compared to the intact controls in only one experiment (March). These results indicate that the pineal, and to some extent the eyes, promotes the occurrence of a daily cycle in serum GTH levels in fish under 16:8D/21° in the spring, although we cannot eliminate the possibility that the results were due to a shift in the phase of the daily cycle. In addition the present results suggest that the pineal and possibly the eyes interdependently stimulate ovarian development in fish under these conditions; the GSIs in the intact and sham control groups were greater than in the pinealectomized groups, a blind-pinealectomized group (March), and a blinded group (April). A stimulatory effect of the pineal on gonadal development under long photoperiod and warm temperature in spring has been previously found in the goldfish, the medaka, and the shiner (see introduction). The effects of blinding on gonadal development reported here are consistent with data reported by Urasaki (1973) who found that the GSI of blinded and pinealectomized medaka held under 14L:10D/21° in the spring was lower than the GSI of the intact fish under the same conditions. Also, in blind medaka held under natural photoperiod in May and June,

the GSI was lower than in intact fish (Urasaki, 1976). However, since pinealectomy more consistently caused reduced GSIs than blinding in our experiments, it seems that the pineal is more important in this regard. It was previously hypothesized that the daily fluctuations in serum GTH levels are of significance in stimulating gonadal activity (Hontela and Peter, 1978; Peter and Crim, 1979). The present results, demonstrating that pinealectomy, and to a lesser extent blinding, of mature fish under 16L:8D/21° in the spring apparently abolishes the daily cycle of serum GTH levels and decreases gonadal size, support this hypothesis.

de Vlaming and Vodcnik (1977) reported that the GTH activity per pituitary was minimal 4 hr and maximal 10 hr after the onset of light in sham-operated female shiners held under 15.5L:8.5D/15° in March, and that pinealectomy abolished such fluctuations and caused decreased gonad size. If it is assumed that a decrease in pituitary GTH activity reflects high serum GTH levels, these results are consistent with data from the present study. Vodcnik *et al.* (1978) reported that pinealectomy abolished the daily cycle in pituitary GTH levels and lowered serum GTH levels early in the day in female goldfish held under 15.5L:8.5D/22° for 22 days in March. The results of Vodcnik *et al.* (1978) also indicate that the serum GTH levels of both the sham-operated and pinealectomised fish had significant daily variations, the levels in both groups being lower at 4 hr than at 10 hr after the onset of light. The finding that the serum GTH levels are higher late in the photophase are not consistent with our present data, or the data reported by Breton *et al.* (1972) and Hontela and Peter (1978) for goldfish subjected to a similar environmental regime. The difference in the timing of the apogee in serum GTH in the two studies could be due to a difference in the preacclimation conditions or in the length of exposure to the

environmental regime. The length of the acclimation period may influence the pattern of daily cycles in serum GTH (Hontela and Peter, unpublished).

The pineal seems to be involved in suppression of the daily cycle in serum GTH levels in mature female goldfish held under 8L:16D/21° in March. No significant differences were detected between serum GTH levels at 1200 and 2000 hr in the sham-operated and the blinded groups, but the levels of the pinealectomised and the blind-pinealectomised fish were significantly higher at 1200 hr than at 2000 hr. However, there were no significant differences between the various groups at 12 or at 2000 hr. de Vlaming and Vodcnik (1977) also reported that pinealectomy had a stimulatory effect on pituitary GTH activity in the shiner held at 9L:15D/15°; significant daily variations in pituitary GTH activity were found in both the sham-operated and the pinealectomised fish, but the magnitude of the variation seemed greater in pinealectomised fish than in the shams. Vodcnik *et al.* (1978) measured plasma and pituitary GTH levels in sham-operated and pinealectomised female goldfish under 8L:16D/21° in March. Fish were sampled once after either 14 or 21 days of exposure to the environmental regime and several pinealectomised fish spawned by Day 21. Although these results suggest a stimulatory effect of pinealectomy on GTH secretion, the evaluation of the data reported by Vodcnik *et al.* (1978) is difficult since Stacey *et al.* (1979) showed that ovulation in the goldfish is accompanied by dramatic changes in serum GTH levels. Although we have not found an effect of pinealectomy on the GSI, reversal by pinealectomy of the inhibitory effect of short photoperiod on gonadal activity in spring has been reported for the shiner, the medaka, and the goldfish (see introduction). Results from the present study, and the literature, demonstrating that pinealectomy of mature fish under short photoperiod and warm conditions in



the spring promotes the occurrence of a significant daily cycle in serum GTH levels and increased gonadal activity, further supports the hypothesis that daily cycles in serum GTH levels are of importance in stimulating gonadal activity (Hontela and Peter, 1978; Peter and Crim, 1979).

Our results also show that blinding alone does not promote a daily cycle in serum GTH levels under short photoperiod in spring, whereas pinealectomy does. This suggests that under short photoperiod, the pineal influence is independent from the eyes, in contrast to the situation under long photoperiod where the effect is interdependent. The only information concerning the effect of blinding under short photoperiod has been provided by Urasaki (1973, 1976) who showed that in the medaka, blinding and pinealectomy have a similar effect on the GSI under short photoperiod. Since these results are not consistent with results reported here, further investigations are required.

Evidence presented in this study indicates that the pineal organ is important in the control of GTH secretion and gonadal development in the goldfish. In the spring the pineal seems to stimulate gonadal development by promoting a daily cycle in serum GTH levels under long photoperiod and to suppress gonadal development by inhibiting such a cycle under short photoperiod. On the other hand, it does not have an apparent effect on GTH levels or gonadal development under long photoperiod in the fall. However, it remains to be shown conclusively whether the pineal receives photic information from the eyes or if it functions independently from the retina. Hafeez *et al.* (1978) demonstrated that while the receptor cells in the pineal of rainbow trout were responsive to photic input through the pineal region, the supporting cells in the pineal were influenced only by photic input from the eyes. This evidence indicates a functional relationship between the pineal and the eyes. Recently,

the retina of rainbow trout has been demonstrated to synthesise melatonin (Gern and Ralph, 1979). Thus, melatonin may be released by the pineal and retina on coordinated or independent cycles and, possibly, these cycles are underlying the independent or interdependent effects of blinding and pinealectomy under various environmental regimes.

Our understanding of the progonadotropic and antigonadotropic effects of the pineal in teleosts remains however limited. Evidence suggesting that melatonin has an antigonadotropic effect in teleosts has accumulated (Fenwick, 1970c; Urasaki, 1972; Sundararaj and Keshavanath, 1976; Saxena and Anand, 1977). However, the basis for the progonadotropic effect of the pineal in teleosts is less well understood. Pro- and antigonadotropic effects of the pineal have been also reported in mammals (Herbert, 1971; Hoffman and Kunderling, 1975) and new avenues of research are opened by some recent studies which have demonstrated that the effects of melatonin on reproductive activity of mammals can be pro- or antigonadotropic depending on the time of the day at which melatonin was administered (Tamarkin *et al.*, 1976) and on the experimental photoperiod regime (Turek *et al.*, 1975; Turek and Loose, 1978). This may suggest that synergistic or antagonistic actions of melatonin with other compounds at various times of the day may result in either pro- or antigonadotropic effects. The present study indicates that, whatever the mechanism involved, the pineal and in some cases the eyes, influence the daily cycles of secretion of GTH in teleosts and that these actions are, at least in part, the basis for the alterations in gonadal activity.

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